PHARMACOLOGICAL MANIPULATION OF OVARIAN FOLLICULAR FUNCTION IN CATTLE

By

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Dedicated to Amy

and

Annie, Alain, Marie-Christine and Isabelle Schmitt

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Abstract of Dissertation Presented to the Graduate School
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PHARMACOLOGICAL MANIPULATION OF OVARIAN FOLLICULAR FUNCTION IN CATTLE

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A series of experiments evaluated use of gonadotropin releasing hormone-agonist (GnRHa; Buserelin, 8 µg), human chorionic gonadotropin (hCG, 3000 IU) and Prostaglandin (PG) $F_{2\alpha}$ (25 mg) to regulate follicle and corpus luteum (CL) function in cattle. GnRHa eliminated a potential persistent follicle and improved conception rates. An accessory CL was induced with administration of GnRHa or hCG on day 5 after estrus and increased plasma progesterone. The increase in progesterone was greater in hCG treated heifers due to enhanced luteinizing hormone (LH)-like stimulation of the preovulatory follicle and subsequent development of the CL. Two fertility experiments indicated that injection of hCG on day 5 after insemination did not improve conception rates in heifers or lactating dairy cows.

Origin of the increase in plasma progesterone following injection of GnRHa or hCG was determined by removing the induced CL on day 13. After lutectomy of accessory CL, progesterone declined to the same level as control cows (saline injection on day 5, sham surgery on day 13) indicating the accessory CL as the source of progesterone. In vitro steroidogenic activity and weights were greater in hCG induced CL. Micromorphometric characteristics of small and large luteal cells differed in the CL induced by hCG, and the development process of steroidogenic luteal cells was reported. To test whether differences in LH priming of a preovulatory follicle altered subsequent CL development, the day 5 dominant follicle was primed with a high or low frequency of LH pulses before induction of ovulation with GnRHa. Preovulatory priming with a high frequency of LH pulses appeared to be critical for ovulation and induction of a functional CL.

A protocol to inseminate heifers at a fixed time was developed and consisted of administering GnRHa at a random stage of the estrous cycle (day 0), $PGF_{2\alpha}$ on day 7, GnRHa on day 9, and insemination on day 9 + 15 h. This treatment sequence did not allow adequate final maturation of the ovulatory follicle and resulted in a high frequency of short luteal phases. Replacing GnRHa on day 9 by hCG eliminated short luteal phases and improved pregnancy rates.

CHAPTER 1
INTRODUCTION

Since the advent of artificial insemination, new technologies have allowed rapid progress in the field of reproduction management. Artificial insemination, embryo transfer and more recently in vitro fertilization techniques have opened new doors to improve desirable genetic traits in dairy and beef cattle. However, to fully benefit from the progress obtained in the field of manipulation of gametes for the improvement of bovine genetics, a clear understanding of the physiology of the bovine estrous cycle and its control is essential.

Modern research techniques allow a more comprehensive investigation of the estrous cycle and its regulation. More specifically, sensitive radio-immunoassay techniques led to a more detailed evaluation of the hormonal changes occurring during the estrous cycle in cattle. Recently, the use of real time ultrasonography to monitor ovarian follicular and luteal function in cattle (Pierson and Ginther, 1984) proved to be a powerful technique to investigate gonadal function in heifers, cows and horses (Pierson et al., 1988). The combination of these two techniques brought a wealth of new knowledge to the scientist and veterinarian interested in the reproductive processes of cattle.

A limited number of products are available to manipulate reproductive function in cattle and all of them are hormonal in nature (Chenault, 1992). However, the uses of this inventory could be expanded with a thorough investigation of the effects of each pharmaceutical agent and the use of several drugs in combination.

Objectives of this study were to evaluate the use of a gonadotropin releasing hormone (GnRH)-agonist, human chorionic gonadotropin (hCG) and prostaglandin (PG) $F_{2\alpha}$ to regulate follicle and corpus luteum (CL) function in cattle and to investigate new protocols to improve reproductive efficiency in cattle. New methods for synchronization of estrus and potential techniques to enhance fertility should enhance progress made in the field of gamete manipulation and accelerate the rate of genetic progress to increase milk and meat production world wide.

CHAPTER 2
LITERATURE REVIEW

Estrous Cycle and Follicular Dynamics

Major functions of the ovary are to generate a mature and fertilizable ovum and then provide the hormonal milieu to maintain a pregnancy until the fetus is sufficiently mature for post-natal survival. The follicle is sometimes described as the housing system of the ovum. In cattle, ovarian follicles follow specific patterns of growth. At birth in calves, a pool of 40,000 to 80,000 quiescent primordial follicles is present and this figure is reduced to approximately 3,000 by 15 to 20 years of age (Erickson, 1966). In adult cattle, during the estrous cycle a large number of antral follicles (50 to 400) are present in the ovary during the estrous cycle (Rajakoski, 1960). However, ovulation rate at each estrous cycle is controlled precisely, since only one follicle will ovulate and the others undergo atresia.

Direct and Histological Observation of Follicular Dynamics

Rajakoski (1960) was the first to described two phases or periods of synchronous follicular growth based on direct and histological observations of ovaries collected at different stages of the estrous cycle. One period occurred from day (d) 3 to d 12 after estrus and the second from d 12 to estrus. Further studies involving marking or cauterization of follicles confirmed these results (Dufour et al., 1972; Matton et al., 1981). Matton et al. (1981), using India ink to mark follicles, identified three periods of follicular growth in cattle that occurred between d 3 and 13, 13 and 18, and 18 and ovulation.

Follicular Steroidogenesis and Follicular Dynamics

Steroidogenesis, and particularly the production of estradiol, is vital to the growth and development of large antral follicles, and for triggering physiological events such as luteolysis, estrus, the preovulatory gonadotropin surge and ovulation (Ireland, 1987). Steroidogenic enzyme activity in theca and granulosa cells is stimulated by LH and FSH after specific receptor binding and activation of cAMP-dependent (intracellular signaling) processes, and the induction of steroidogenic cytochrome P450 enzymes (Richards and Hedin, 1988). Follicle stimulating hormone binds to granulosa (but not to theca) cells at all stages of follicular development, whereas LH binds to theca and luteal cells; LH also binds to granulosa cells, but only in large preovulatory follicles after the induction of LH receptors by the combined action of FSH and estradiol (Richards, 1980; Ireland and Roche, 1983a,b). The response of ovarian cells to gonadotropin is not only dependent upon the levels of these hormones in blood, but also in the content of their receptors in target cells. Moreover, LH and FSH can up- or down-regulate the membrane concentrations of their own receptors or the presence of receptors for other hormones. In addition, LH and FSH receptor binding depends on the functional coupling of the ligand-receptor complex to intracellular signalling systems, and may be modulated at the post-receptor level by the actions of other endocrine, paracrine or autocrine factors, particularly those of estradiol (Richards & Hedin, 1988).

The mechanisms of action of LH and FSH on theca and granulosa cells have been extensively reviewed (Richards, 1980; Richards and Hedin, 1988; Fortune and Quirk, 1988). In theca cells, LH promotes the synthesis of androgens (predominantly androstenedione in the cow) by induction of cholesterol side-chain cleavage P450 and 17α-hydroxylase P450 enzyme activity which converts cholesterol to pregnenolone and then to androstenedione. The granulosa cell is dependent upon the theca for provision of androgen substrate for aromatization to estradiol by the aromatase P450 enzyme under the stimulation of FSH. Estradiol, in turn, plays an integral role in regulating gene expression within the granulosa cell, increasing transcription of the LH receptor, components of the cAMP second-messenger system and cytochrome p450 enzymes, but can only exert these effects in the presence of FSH. In bovine follicles, estradiol produced by the granulosa cells also exerts positive feedback effects on theca cell androstenedione production, and inhibits the secretion of progesterone by both theca and granulosa cells (Fortune and Quirk, 1988). Thus, steroidogenesis in ovarian follicles involves the integral communication and interaction between these cells types; androgens produced by theca cells are used as a substrate for granulosa cell production of estrogens, which in turn feed back to stimulate theca cell production of androgens. In particular, the synthesis of estradiol and its local actions on both cell types appears to be essential for the growth and maturation of preovulatory follicles (Richards and Hedin, 1988). Regulation of this two cell two gonadotropin model was described by Fortune and Quirk (1988).

Ireland and Roche (1983a,b) classified follicles as "estrogen-active" or "estrogen inactive" based on the ratio of the absolute concentrations of estradiol to progesterone in follicular fluid. Follicles that had a higher concentration of estradiol than progesterone were classified as estrogen-active. This classification was used as an index of follicular atresia since follicles with a higher concentration of progesterone than estradiol are considered atretic (McNatty, 1979; Ireland and Roche, 1982). Ireland and Roche (1983b), in agreement with results from Matton et al. (1981), defined two periods of follicular growth from d 3 to 7 and d 7 to 13. Measurement of estradiol in serum of cows (Lukaszewska and Hansel, 1980) showed concentrations from d 3 to d 5 of the cycle, that were comparable to values at estrus. This confirmed the hypothesis that non-ovulatory estrogen-active follicles develop during early stages of the estrous cycle. Glencross et al. (1973) also reported a rise in concentration of estradiol in blood that occurred after estrus, whereas Hansel and Echternkamp (1972) observed another increase in estradiol at mid-cycle. While these results tended to confirm that two phases or waves of follicular growth occurred in cattle, analysis of utero-ovarian venous blood samples proved to be a more precise way to monitor differential ovarian follicular function and hierarchy in cattle (Ireland et al., 1984; Fogwell et al., 1985). Ireland (1987) proposed that the terms defined by Goodman and Hodgen (1983) for primates could describe the process of folliculogenesis in cattle. For example, recruitment is a gonadotropin-dependent event during which a group of follicles gain the ability to respond to gonadotropins and require gonadotropin for their growth. Selection is a process whereby only a single follicle of the recruited follicular pool is selected to escape atresia and survived to ovulate. Dominance is the mechanism that a selected follicle uses to escape atresia and block recruitment of a new follicular wave.

Symmetrical production of estradiol from both ovaries as measured in the utero-ovarian venous blood samples, indicates that the selection process for a dominant follicle is on-going, whereas asymmetrical production of estradiol indicates that the selection process is completed and dominance has been established. Based on these measurements, Ireland and Roche (1987) suggested the existence of three periods of follicular growth in cattle.

Ultrasound Imaging and Follicular Dynamics

The advent of real time ultrasound imaging and development of rectal probes for use in horses or cattle (Pierson and Ginther, 1984) brought a definitive answer to the phenomenon of follicular growth in cattle. Ultrasonographic scanning of ovaries is a rapid, non-invasive, repeatable technique to monitor follicular dynamics and corpus luteum (CL) development in cattle. Pierson and Ginther (1987a,b,c), on the basis of counts of follicles of different sizes, suggested that two follicles of ovulatory size develop in cattle during the estrous cycle. One follicle grew during diestrus and the other during the proestrous period. In addition, they provided evidence that the proestrous follicle becomes dominant 3 to 4 d before ovulation. When ultrasonography was used to follow the pattern of development of individual follicles of size greater than 5 mm in diameter, the existence of two to three periods of follicular development (Savio et al., 1988; Sirois and Fortune, 1988; Ginther et al., 1989d) was demonstrated during the estrous cycle of heifers. Each wave is comprised of a recruitment phase with the appearance of three to six follicles whose diameters are greater than 5 mm (recruitment). One of these follicles becomes larger than the rest (selection) and continues its growth (dominance) while the other follicles in the recruited cohort regress (atresia).

Ginther et al. (1989a,b) observed that cattle with three follicular waves had longer inter-ovulatory intervals and luteal phases than cows with two follicular waves. Ginther et al. (1989b) defined the dominant follicle as the follicular structure that grew to at least 11 mm in diameter and exceeded the diameter of all other follicles within a wave. Sirois and Fortune (1990) defined the dominant follicle as a follicular structure whose diameter is 2 mm greater than other follicular structures of the same wave. This definition is utilized for the studies of this dissertation. In a two wave pattern, the dominant follicle of wave one can be identified on d 3 to 4 and reaches a diameter of 16 mm on d 6 or d 7. Dominance is established on d 5. This follicle remains static for 6 d (until d 9 to d 12 of the estrous cycle) and then regresses in size and function. The second wave is detected on approximately between day 9 to d 14 (Ginther et al., 1989c; Savio et al., 1993a) and gives rise to a dominant follicle that will become the ovulatory follicle. For both waves, subordinate follicles stop growing 3 d after first detection of a wave (Ginther et al., 1989c).

In cows having three waves of follicular growth, the first wave dominant follicle is detected on d 4 after estrus, reaches its maximum size on d 6 and continues in a plateau phase until d 10. This first wave dominant follicle is no longer detectable on d 15. The second dominant follicle emerges on d 9 and reaches its maximum size on d 16. The third dominant follicle (ovulatory follicle) is usually detected on d 16 and its maximum size is reached on d 21 (Savio et al., 1988).

In a two wave pattern of follicular growth, the ovulatory follicle will emerge 11 d before ovulation, whereas in the case of a three wave pattern, the interval from follicle emergence to ovulation is only 7 d (Ginther et al., 1989c). Perhaps the extended interval from emergence of the last follicular wave of the estrous cycle to ovulation (11 d) in cows presenting two waves of follicular growth when compared to cows presenting three waves (7 d) could alter fertility upon ovulation.

There is no clear explanation for the differential occurrence of two or three follicular waves in cattle. Some studies reported a preponderance of two wave estrous cycles (Ginther et al., 1989a,c) while others observed a greater frequency of three wave cycles (Savio et al., 1988; Sirois and Fortune, 1988). Variation was attributed to differences in season (Badinga et al., 1995) or energy balance (Lucy et al., 1992). Estrous cycles comprised of only one wave (Savio et al., 1988) or a maximum of four waves (Sirois and Fortune, 1988) also are described but are uncommon. Patterns of growth for the first wave dominant follicle are identical in cattle exhibiting two or three waves of follicular development.

Follicular waves have been described in cattle in different reproductive states. In prepubertal heifers, the successive development and regression of large follicular structures in the absence of high concentrations of progesterone was observed (Roche and Boland, 1991). During early pregnancy, periods of growth of dominant follicles occurring at an interval of 10 to 12 d, were recorded (Ginther et al., 1989c; Savio et al, 1990c). Thatcher et al. (1991) reported an attenuation of follicular growth on the CL bearing ovary during early pregnancy (d 17 to d 34) and suggested the existence of compounds secreted by the conceptus and/or uterus that would alter follicular development. The loss of follicular estrogenic activity at the time of pregnancy recognition on the ovary ipsilateral to the uterine horn bearing an embryo may contribute to the antiluteolytic effect of early pregnancy. For dairy and beef cows in the post-partum period (Roche and Boland, 1991), resumption of follicular activity was first characterized by the development of follicles from 5 to 8 mm in diameter until emergence of a dominant follicle on d 11.6 ± 8.9 post-partum for dairy cows and d 10.2 ± 2.2 post-partum for beef cows. In beef and dairy cows, when the first dominant follicle ovulates after d 20 post-partum, the following estrous cycle is short (9 to 13 days). In contrast, dairy cows developing ovulatory follicles earlier in the post-partum period (before d 20) had estrous cycles of normal or extended length. Perhaps length of the first estrous cycle after parturition is influenced by a carryover effect of pregnancy. In lactating dairy cows, the first post-partum dominant follicle ovulated on d 27.4 ± 18.9 in 74% (14/19) of the animals (Savio et al., 1990b). This is probably due to the resumption of a pulsatile pattern of luteinizing hormone (LH) secretion early after parturition in lactating dairy cows that can support follicular maturation and ovulation (Peters and Lamming, 1984). In contrast, the interval from calving to first ovulation is extended in post-partum beef suckler cows (35.6 ± 3.3 days, Murphy et al., 1990; 82 ± 4.7 days, Perry et al., 1991), and several waves of follicular growth occur before the first ovulation (Murphy et al., 1990). This extension was attributed to the inhibitory effect of suckling on LH secretion reported in post-partum beef cows (Peters and Lamming, 1984; Williams, 1990; Murphy et al., 1990).

A precise description of the wave-like pattern of follicular development in cattle explains variability observed with certain estrus synchronization protocols as well as variations observed in response to superovulation. For instance, if luteolysis is initiated at the end of a dominance phase when the dominant follicle is undergoing atresia, the interval to estrus is extended because a new dominant follicle must grow and become estrogenic before inducing estrus and the surge of LH. However, the interval is short when luteolysis is induced at the beginning of a period of dominance (d 5 to d 7 of the estrous cycle) when the dominant follicle is fully functional (Tanabe and Hann, 1984; Macmillan and Henderson, 1984; Savio et al., 1990a). Similarly, if superovulation is initiated during a period of follicular dominance, the FSH induced recruitment will be inhibited by the presence of a dominant follicle (Adams, 1994). Therefore, superovulatory responses are improved if superovulation is initiated between d 9 to d 12 of the estrous cycle (during the regression phase of the first wave dominant follicle) or at a time when the absence of a dominant follicle can be confirmed by ultrasonography (Guilbault et al., 1991; Bungartz and Niemann, 1994).

Control of Follicular Waves in Cattle

There is a large variation in the number of primordial follicles between animals and the pool decreases with increasing age. Antral follicles will develop from this pool of non-proliferating follicles. Antrum formation in cattle occurs when follicles reach the size of 0.8 mm (Lussier et al., 1987). It takes a further 40 days or two estrous cycles for the antral follicle to reach ovulatory size (Lussier et al., 1987). It is generally accepted that pre-antral and early antral follicular growth in the ewe and cow proceed at least partly in the absence of LH and follicle stimulating hormone (FSH). However, some reports suggest that pregnant mare serum gonadotropin (PMSG) could alter the proportion of pre-antral follicles (Monniaux et al., 1984). In this experiment heifers were treated with PMSG immediately after unilateral ovariectomy. The remaining ovary was removed 48 h after treatment and was compared to the ovary removed at the time of PMSG administration. Each animal served as its own control. An increase in the mitotic index of preantral and early antral follicles was observed while atresia in large antral follicles (> 1.7 mm) was reduced, suggesting some "rescue" of antral follicles after PMSG treatment. Perhaps the effect of PMSG observed by Monniaux et al. (1984) was mediated through the action of another factor secreted by the large antral follicles presenting FSH receptors.

Activin supports granulosa cell differentiation and production of FSH receptors and may be of particular importance in the mechanism by which preantral and early antral follicles become responsive to FSH (Findlay, 1993). Activin could also be involved in the "rescue" of large antral follicles (> 1.7 mm) observed by Monniaux et al. (1984) since activin can prevent luteinization of differentiated granulosa cells in vitro (Findlay, 1993). The recruitment of primordial follicles in the transitory pool is an important process since it determines when and how many follicles subsequently enter the growing pool. However, very little is known about the mechanisms involved in this process. Once a follicle begins to grow, growth seems to be continuous until the follicle meets one of two fates: ovulation or atresia (Fortune, 1994).

Receptors for FSH are expressed in follicles < 4 mm (Wandji et al., 1992). Small antral follicles contain FSH receptors and not LH receptors, and they are located on the granulosa cells. In large antral follicles, LH receptors are on the theca cells, and both LH and FSH receptors are on granulosa cells (Ireland and Roche, 1982). Follicular growth becomes gonadotropin dependent when the follicle reaches the size of 2 mm in sheep and 4 mm in cattle (Driancourt, 1991; Lussier et al., 1994, Campbell et al., 1995).

Histological reports in sheep suggest that pituitary hormones are necessary to maintain the rate at which follicles enter the growth phase and develop. Ewes that were hypophysectomized for 70 days had decreased antral development (Dufour et al., 1979). Two days after hypophysectomy in sheep, development of antral follicles > 2 mm is prevented (Driancourt et al., 1987).

Inhibition of the pituitary release of gonadotropins can be achieved by continuous infusion or repeated injection of a GnRH-agonist. In sheep 39 days of continuous infusion of a GnRH-agonist induced pituitary desensitization to GnRH and the ovary did not bear follicles larger than 2.5 mm in diameter (McNeilly and Fraser, 1987). In cattle, when a potent GnRH-agonist was injected twice daily for 21 days, gonadotropic and follicular responses showed two stages (Gong et al., 1995). During the first stage, FSH secretion was maintained but LH secretion was reduced and follicles observed by ultrasonography did not exceed 7-9 mm in diameter. During the second stage, FSH secretion was suppressed and no follicle bigger than 2-3 mm was observed. This supports the hypothesis that growth of follicles from 2 mm to 8 mm is under the control of FSH whereas growth of follicles larger than 8 mm is under the gonadotrophic support of LH.

In sheep, growth of follicles to an ovulatory size is totally dependent on FSH and the number of follicles that develop is dependent on the amount of FSH and the duration of FSH exposure (Picton et al., 1990). Large pre-ovulatory ovine follicles transfer their gonadotrophic requirements from FSH to LH secretion if LH is delivered as high frequency low amplitude pulses. These large follicles become critically dependent on LH for support, and cessation of high LH pulse frequency will lead to atresia. Campbell et al. (1995) hypothesized that this transfer of gonadotropic requirements may be the mechanism by which follicles escape atresia in sheep. This is supported by results of Glencross (1987) in which an increase in follicular development and estradiol secretion occurred when heifers were treated with hourly infusions of gonadotropin releasing hormone (GnRH) to elevate plasma LH during the luteal phase of the estrous cycle.

Savio et al. (1993a) demonstrated that a dominant follicle of cattle can be rescued from atresia when maintained in a low progestin environment. In the presence of a low progesterone or progestin environment, LH pulsatile frequency increased and growth of the dominant follicle was maintained for 15 to 20 days (Lucy et al., 1990; Sirois and Fortune, 1990; Savio et al., 1993a). During this period of extended dominance, follicular recruitment did not occur (Sirois and Fortune, 1990; Lucy et al., 1992; Savio et al., 1993a). The high frequency / low amplitude pulses of LH observed when a low progesterone environment is maintained sustain growth and dominance of the first wave dominant follicle, whereas low frequency and high amplitude LH pulses during the mid luteal phase (Rahe et al., 1980) of high progesterone secretion result in turnover of the dominant follicle and initiation of a new follicular wave (Savio et al., 1993a).

Ireland and Roche (1987) suggested that FSH may be the stimulus for recruitment and subsequent selection of a dominant follicle. Destruction of the dominant follicle by cautery on d 3 or d 5 after ovulation in heifers resulted in a surge of FSH beginning the day after cautery (Adams et al., 1992b). Heifers having two or three waves of follicular growth during the estrous cycle were shown to have two or three FSH surges, respectively. Each FSH surge occurred 2 to 3 days before emergence of the respective follicular waves as detected by ultrasonography (Adams et al., 1992b; Sunderland et al., 1994). The surge peaked 1 or 2 days before emergence of the follicular wave and began to decrease when the size of the dominant follicle started to diverge from the first subordinate follicle. Badinga et al. (1992) demonstrated that removal of the ovary bearing the first wave dominant follicle was followed by a surge in FSH and an increase in the number of follicles in the 3-4 mm class. FSH would stimulate growth of follicles (recruitment) beyond the 3 mm stage whereas a decrease of plasma FSH induced by the dominant follicle would initiate atresia in subordinate follicles > 3 to 4 mm (Lussier et al., 1994).

In summary, recruitment of a follicular wave is triggered by a surge of FSH. Growth of follicles until selection seems to be under the main control of FSH. Final growth of the dominant follicle is dependant on LH support. The amplitude and frequency of LH pulses will allow the final development of an ovulatory follicle (pulses of high frequency and low amplitude) or will induce follicular atresia and turnover (pulses of low frequency and high amplitude). Inhibition of FSH secretion by the dominant follicle probably induces atresia of subordinate follicles that did not reach a maturation stage where their growth could be sustained only by LH.

Dominance and Intraovarian Control of Follicular Development

There is strong evidence that follicular development is modulated through the action of inhibitory and stimulatory factors secreted at the level of the ovary. These factors alter the pattern of gonadotropin release but also follicular responsiveness to gonadotropins (Ireland, 1987; Adashi, 1994).

Staigmiller and England (1982) removed the ovary with the largest follicle or the ovary contralateral to the largest follicle and assessed the remaining ovary 4 days later. Removal of the ovary with the largest follicle resulted in an increase in follicular size and follicular fluid weight of the remaining ovary but removal of the contralateral ovary did not. Other studies utilizing unilateral ovariectomy (Badinga et al., 1992) and follicular cauterization (Matton et al., 1981; Ko et al., 1991; Adams et al., 1992b) demonstrated that a single large follicle (dominant follicle) inhibits the development of other small follicles. The effect of steroid free bovine follicular fluid as a source of inhibins has been intensively studied in cattle. Injection of charcoal stripped bovine follicular fluid selectively reduced concentrations of FSH in plasma of intact (Miller et al., 1979; Moser et al., 1989; Turzillo and Fortune, 1990) and ovariectomized (Beard et al., 1989) cattle as well as the secretion of FSH by pituitary cells in culture (Lussier et al., 1993). Injection of charcoal stripped bovine follicular fluid (steroid free) also prevented growth of a dominant follicle (Kastelic et al., 1990b; Turzillo and Fortune, 1990). The population of antral follicles smaller than 3.7 mm was not affected by 12 days of treatment with charcoal stripped bovine follicular fluid that dramatically reduced circulating concentrations of plasma FSH (Lussier et al.,1994). Since follicles smaller than 4 mm express the FSH receptor (Wandji et al., 1992), small antral follicles may be less susceptible to low FSH concentrations because of differences in the structure or coupling of the receptor.

Due to the numerous forms of inhibins and the absence of a specific assay to measure biologically active molecules (Ireland et al., 1994), the negative association of circulating concentrations of FSH and inhibins cannot be demonstrated.

In addition to their role as circulating inhibitors of FSH secretion, strong evidence supports a local role for inhibins. Early reports from Sato et al. (1978) demonstrated that a substance secreted by porcine granulosa cells could inhibit FSH activity at the ovarian level in mice. Further experiments allowed purification of an inhibin-like molecule that prevented the binding of FSH to granulosa cells (Sato et al., 1982). More recently, Schneyer et al. (1991) reported that an α-inhibin subunit from different sources (porcine and human) antagonized FSH binding to its receptor in bovine tissue culture as well as its biological activity. Furthermore, the specific activity of α-inhibin subunit is suggested by an absence of binding to LH receptors.

In summary, inhibins are important factors by which the dominant follicle suppresses growth of subordinate follicles. Inhibins exert their actions as classic endocrine hormones by inhibiting FSH release from the pituitary and probably as a direct local regulator of FSH activity. Other substances of the inhibin family and of a proteinaceous nature are present in charcoal stripped (steroid free) bovine follicular fluid and exert an action on the process of folliculogenesis (for review see Findlay, 1993). This information should be kept in mind and suggests a poor specificity of steroid free bovine follicular fluid as used in several experiments described previously.

Steroid Control of Gonadotropin Secretion

Gonadotropins are synthesized by the gonadotropes of the pituitary. Messenger ribonucleic acid (mRNA) coding for the beta subunits of LH and FSH were detected in the same cells in porcine pituitaries (Liu et al., 1988). Therefore, both LH and FSH are secreted by the same cells. Differential glycosylation of FSH and LH may direct the gonadotropins to different secretory granules which in turn enables differential release of FSH and LH.

Luteinizing hormone is released under GnRH stimulation from bovine pituitary cells in vitro (Padmanabhan et al., 1978; Baratta et al., 1994) and in vivo (Thatcher and Chenault, 1976). Follicle stimulating hormone also is released in vivo after administration of GnRH (Chenault et al., 1990a). The basal secretion of LH and FSH requires the continuous presence of GnRH.

Luteinizing hormone release is influenced by a direct effect of gonadal steroids on gonadotropes. Baratta et al. (1994) demonstrated that progesterone alone did not influence LH release, but negatively affected GnRH-induced LH release in vitro. Estradiol enhanced in vitro release of LH after 15 h of exposure. When progesterone is added to the culture medium containing estradiol, the increased release of LH induced by estradiol alone is abolished. Finally, GnRH-induced release of LH was modulated by estradiol in a time dependent manner, with an initial inhibitory effect, then stimulatory and finally inhibitory again (Baratta et al., 1994). This is confirmed by the in vivo observations of Kesner et al. (1981) that in steers, increasing the serum concentrations of estradiol first reduced, then increased the ability of the pituitary to release LH in response to GnRH.

Steroid Control of Gonadotropin Secretion During the Peri-Ovulatory Period

Increasing concentrations of estradiol in plasma after the decline in plasma progesterone will induce a preovulatory surge of LH in cows (Kesner et al., 1981). Estradiol may initiate the preovulatory surge of LH by acting on the hypothalamus to increase secretion of GnRH and/or at the pituitary level to increase the sensitivity of the gonadotropes to GnRH. Imakawa et al. (1986) proposed the following model: decline in progesterone initiates the preovulatory events in cattle by permitting an increase in the follicular response to LH. The decline in progesterone also increases the frequency of LH pulses which stimulates estradiol secretion by the follicles. The increase in estradiol in turn maintains the amplitude of LH pulses. Therefore, the increase in LH release occurring at the time of estrus is due to a combined effect of a progesterone decrease and an increase in circulating estradiol. Although the release in LH is stimulated by estradiol during the follicular phase of the estrous cycle, the amount of mRNA for gonadotropin subunits in the pituitary is reduced (Cupp et al., 1995b). Recent studies in sheep (Karsh et al., 1992) with intensive sampling of blood from the hypophyseal portal system, reported that the pre-ovulatory surge of LH is induced by a massive and sustained release of GnRH.

It is difficult to generalize about the circulating concentrations of FSH in cattle due to the wide disparity between results. During the period preceding the LH peak, a decrease in FSH was reported (Schallenberger et al., 1984, Quirk and Fortune, 1986). A pre-ovulatory surge of FSH occurring concomitantly with the surge of LH was reported in cattle (Walters and Schallenberger, 1984; Quirk and Fortune, 1986; Cupp et al., 1995a). Therefore, during the pre-ovulatory period estradiol will have first an inhibitory effect than a stimulatory effect on FSH stimulation. Perhaps the inhibitory effect of estradiol is overcame by the massive release of GnRH occurring at the time of the pre-ovulatory surge of gonadotropin. A massive release of FSH also was reported 4 to 12 h after the surge of LH (Walters and Schallenberger, 1984), and this increase is probably due to the decline in circulating inhibin after luteinization of the ovulatory follicle (Adams et al., 1992b). No release of LH for 12 h after the surge of LH was detected in cattle (Walters and Schallenberger, 1984). This is probably due to a desensitization of the pituitary to GnRH. In ewes, GnRH secretion remains elevated for several hours after occurrence of the pre-ovulatory surge of LH (Karsh et al., 1992). Down-regulation of pituitary receptors for GnRH was induced after infusion of GnRH for 16 h (Nett et al., 1981). This is confirmed in cattle by a decrease in GnRH receptors in the pituitary of cycling heifers after estrus (Schoenemann et al., 1985). Cows will respond to a stimulatory injection of GnRH given 20 to 30 h after a spontaneous preovulatory surge of gonadotropins with an additional release of LH of lesser amplitude (Lucy and Stevenson, 1986).

Steroid Control of Gonadotropin Secretion During the Luteal Period

During the luteal phase of the estrous cycle, LH secretion is controlled by the circulating concentrations of progesterone or progestins (Savio et al., 1993b). In a high progesterone environment LH will be released with a low frequency and high amplitude pattern (Rahe et al., 1980, Schallenberger et al., 1984; Cupp et al., 1995a). To test the direct effects of progesterone on the release of GnRH and LH in bovine, Kawate et al. (1993) isolated and perifused tissues of the pituitary stalk-median eminence and anterior pituitary with medium containing a high concentration of progesterone (comparable to what is observed during the luteal phase). The release of GnRH from the hypothalamic fragments was reduced. However, GnRH induced release of LH was significantly higher than what was observed in the control group. This confirmed the in vivo observation of Rahe et al. (1980), that observed an LH pattern of high amplitude during the luteal phase.

Knowledge of a negative feed back control of FSH in cattle is scant. Estradiol given as a large single injection will reduce FSH concentrations to precastration values in ovariectomized heifers (Kesner and Convey, 1982).

Ovulation, Formation and Regression of the CL

Changes of LH in the Peri-Ovulatory Period

Ovulation in cattle is triggered by a surge of LH. The pre-ovulatory surge of LH lasts for approximately 10 h (Chenault et al., 1975; Chenault et al., 1976; Thatcher and Chenault, 1976; Rahe et al., 1980; Cupp et al., 1995a). In ewes, sampling of the hypophyseal portal blood system reveals that the preovulatory surge of LH is due to a massive and sustained surge of GnRH triggered by an increase in circulating estradiol (Karsch et al., 1992). Before the pre-ovulatory surge of LH, concentrations of this hormone fluctuate with a high frequency low amplitude pattern that is coupled with an increase in circulating estradiol and a decrease in circulating progesterone (Rahe et al., 1980; Walters et al., 1984, Walters and Schallenberger 1984; Cupp et al., 1995a). The mean concentration of LH increased linearly during the period preceding the pre-ovulatory peak of LH (Chenault et al., 1975, 1976). Growth and differentiation of the ovulatory follicle during the preovulatory stage will dictate the formation of a functional CL (McNatty, 1979). In primates, inappropriate patterns of pituitary gonadotropins in circulation lead to abnormalities in the developing dominant follicle as revealed by deficiency in estrogen secretion and the inability of granulosa cells to luteinize appropriately (DiZerega and Hodgen, 1981). Consequently, variation in gonadotropins recorded during the period preceding the ovulatory surge of LH may play a role in the preparation of the dominant follicle for luteinization and ovulation. Greve et al. (1995) also reported that a reduction in concentrations of circulating LH during the period from luteolysis to the LH ovulatory surge alters maturation of the oocyte.

Few reports have characterized circulating LH concentrations during the period following the ovulatory surge. Walters and Schallenberger (1984) did not detect any pulsatile secretion of LH for 12 h after the ovulatory surge, and mean concentrations of LH were lower than before or during the surge. Sampling of the hypophyseal portal venous system in ewes suggests that the period of low LH concentration following the LH surge is due to a refractory period of the pituitary since secretion of GnRH continues (Karsch et al., 1992). In cattle consecutive injections of GnRH every 4 h for 96 h induced a release of LH after each injection.

However the amplitude and duration of the release of LH was considerably reduced after the first injection (Kinder et al., 1975) and a desensitization of the pituitary to GnRH was suggested. Low-amplitude pulses of LH resumed 12 h after the preovulatory surge. From d 2 to d 4 after detection of behavioral estrus, a pulsatile pattern of LH similar to what is observed after luteolysis (high frequency and low amplitude) is reported (Rahe et al., 1980; Peters et al., 1994; Cupp et al., 1995b). This period of high LH pulsatility is essential to the correct development and maintenance of the CL (Burke et al., 1994; Peters et al., 1994).

The Process of Ovulation and Differentiation of Granulosa and Theca Cells into Luteal Cells

Luteal function and progesterone secretion are essential for regulation of the estrous cycle as well as establishment of pregnancy (Hansel and Convey, 1983; Wilmut et al., 1986; Thatcher et al., 1994). Furthermore, regulation of progesterone or progestin concentrations in plasma has essential implications for controlling the time of ovulation in livestock species (Odde, 1990).

Several questions remain unanswered regarding the process of CL development. The follicular lineage of luteal cells and the mechanisms associated with luteinization are not understood perfectly (Smith et al., 1994). Corpora lutea are the continuation of follicular structures present on the ovary at the time of the preovulatory surge of LH. The preparation of luteal cells for the synthesis of progesterone begins before ovulation (McNatty, 1979, McNatty et al., 1981). After the pre-ovulatory surge of LH, plasma concentrations of estradiol decrease to basal levels within 14 h and before ovulation. Characterization of changes in the micromorphology of the follicular wall and hormonal concentrations of bovine follicular fluid relative to the peak of luteinizing hormone revealed that from 6 to 20 h after the pre-ovulatory surge the level of estradiol in follicular fluid dropped rapidly. At 20 h after the pre-ovulatory surge of LH and before ovulation, concentrations of progesterone in the follicular fluid increased to high levels, concomitantly with an increase in size of the granulosa cells (Dieleman et al., 1983).

The CL consists of an heterogenous population of cells that have distinct morphological, endocrinological and biochemical properties (Niswender and Nett, 1988). Cell types identified in the CL of cattle include small luteal cells, large luteal cells , fibroblasts, endothelial cells and pericytes (Hansel et al., 1991). In cattle, steroidogenic cells are the small and large luteal cells. During the ovine estrous cycle, small luteal cells increase in number but not in size whereas large luteal cells increase in size but not in number (Farin et al., 1986). In cattle, basal progesterone secretion by small luteal cells is less than progesterone secretion by large luteal cells (Urseley and Leymarie, 1979). However, the magnitude of LH-stimulated progesterone secretion is greater in small luteal cells (Hansel et al., 1991). Niswender et al. (1985) calculated that 80 % of the progesterone secreted in vivo by the CL of sheep is secreted by large luteal cells.

The morphology of small and large luteal cells was reviewed by O'Shea (1987) and Wiltbank (1994). Major characteristics of small and large luteal cells are described in Table 1 as adapted from Wiltbank (1994).

Some authors suggest that the distinction between large and small luteal cells is not as easy in cattle as it is in sheep or goats (reviewed by O'Shea, 1987). Fields et al. (1985) outlined characteristics that are specific of bovine small and large luteal cells. In large luteal cells there is an absence of the abundant stack of cisternae of rough endoplasmic reticulum present in small luteal cells. There is a preponderance of lipid droplets in small luteal cells and secretory granules in large

Table 1. Morphological characteristics of cells in the mid-cycle CL of domestic ruminants (adapted from Wiltbank, 1994)

Cell type	Size µm	% of luteal volume	% of luteal cells	Distinguishing Characteristics
Large luteal cells	38	40	3.5	Shape: spherical to polyhedral Nucleus: spherical Rough endoplasmic reticulum: rare Smooth endoplasmic reticulum: abundant Mitochondria: abundant (20% of volume) Secretory granules: abundant / oxytocin (.2 µm electron dense) Plasma membrane: highly folded with microvilli Lipid droplets: rare except during luteolysis Basal lamina: abundant
Small luteal cells	17	28	27	Shape: spindle Nucleus: irregular with cytoplasmic inclusions Rough endoplasmic reticulum: abundant isolated stacks Smooth endoplasmic reticulum: abundant Mitochondria: abundant (15% of volume) Plasma membrane: few microvilli Lipid droplets: present Basal lamina: not well defined

luteal cells. In cattle, secretory granules contain oxytocin (Shams et al., 1987). Molecules having relaxin bioactivity were isolated from corpora lutea of late pregnant cows (Fields et al., 1980) but no definitive cytochemical localization of relaxin was possible in the late pregnant CL. Secretory granule exocytosis occurred under the stimulation of $PGF_{2\alpha}$ in late pregnant cows (Fields et al., 1989).

The follicular lineage of steroidogenic luteal cells has been a topic of investigations for many years. Loeb (1906) performed a detailed morphological analysis of the guinea pig CL and concluded that cells from the granulosa and theca layer of the CL were involved in the formation of the CL. MacNutt (1924) and Gier and Marion (1961), describing the bovine CL after treatment with bovine LH or hCG, came to the same conclusion. Donaldson and Hansel (1965a) reported that luteal cells are derived from theca and granulosa cells. They hypothesized that large luteal cells originating from the granulosa cells do not undergo any further division and therefore have a limited potential. In contrast they suggested that theca cells under the stimulation of plasma luteotropin or hCG multiply, develop into small luteal cells that will form larger luteal cells later in the luteal phase.

In sheep and cattle, the general consensus is that granulosa cells differentiate into large luteal cells and small luteal cells originate from the theca cells (O'Shea, 1987). The fate of granulosa cells was studied utilizing morphological and immunological approaches. Monoclonal antibodies raised against cell surface antigens of granulosa and theca cells have been used as markers for granulosa and thecal derived cells of the CL in cattle (Alila and Hansel, 1984). With this technique, large cells of the early CL were shown to be derived from granulosa cells and small luteal cells were derived from theca cells. These results also indicated that small luteal cells can develop into large luteal cells throughout the estrous cycle and early pregnancy. For example, the percentage of cells binding to the granulosa antibody declined throughout the estrous cycle when the percentage of large luteal cells binding the thecal antibody increased throughout the estrous cycle and early pregnancy. In bovine CL, most of the growth during the 4 days associated with proliferation was due to proliferation of theca originated cells, whereas little cell division was observed for granulosa derived cells (Zheng et al., 1994). In ewes the number of granulosa and theca cells in the preovulatory follicle approximate the number of large and small luteal cells, respectively in the CL (O'Shea et al., 1980; Rodgers et al., 1984). Finally, bovine granulosa and theca cells cultured, in the presence of forskolin and insulin for 9 days can be induced to differentiate into large and small luteal-like cells, respectively (Meidan et al., 1990).

The idea that large luteal cells originate only from granulosa cells remains controversial. Several studies suggest that small luteal cells differentiate into large lutea cells and that there may be a population of stem cells in the CL which differentiate into small luteal cells which in turn differentiate into large luteal cells (Niswender et al., 1985). The presence of cells that are morphologically intermediate between fibroblasts and small luteal cells has been reported in the ewe (O'Shea et al., 1980). Niswender et al. (1985) also reported that the transformation of small luteal cells to large luteal cells could be increased by hCG stimulation in vivo. In a later study, treatment of ewes with pharmacological concentrations of LH induced the formation of cells intermediate between small and large luteal cells (Farin et al., 1988). When ewes are treated with physiological concentrations of LH, these intermediate cells were not observed. A final argument came with observation of follicular cysts formed after treatment with PMSG in ewes. In these follicular cysts, granulosa cells are mostly degenerated. However, after luteinization of the follicular cysts, steroidogenic cells with a structure similar to that of large luteal cells can be observed (Cran, 1983).

If the dual origin of large luteal cells can be confirmed, it remains an open question as to why luteal cells of granulosa origin should be replaced. O'Shea (1987) proposed two hypothesis: 1) the granulosa derived large luteal cells may have a lifespan sufficient for the estrous cycle but not for maintenance of pregnancy or 2) replacement of granulosa cells with theca derived luteal cells may provide the CL with functionally different cells. For instance, replacement may be necessary to provide a cell capable of synthesizing relaxin in place of an oxytocin secreting cell. This leads to a testable hypothesis that individual large luteal cells may secrete either oxytocin or relaxin but not both. However to date relaxin has never be conclusively identified in CL of cattle .

Cellular Control of Steroidogenesis

The preovulatory surge of LH sets into motion a series of biochemical changes that accompanies the morphological changes occurring in follicular cells. These changes switch the steroidogenic metabolism of follicular cells to inhibit synthesis of estrogen and androgen and increase production of progesterone (Fortune, 1994). The activities of cholesterol esterase, 3β-HSD and cytochrome $P450_{scc}$ are essential for progesterone synthesis as the CL became fully functional (Niswender et al., 1994). Receptors for LH on granulosa cells were first down regulated after the pre-ovulatory surge of LH, then total concentration of LH receptors in the ovine CL increased as the CL formed to reach a maximum size on d 10 (Niswender et al., 1985). There is evidence that unstimulated large luteal cells secrete progesterone at a higher rate (2 - 40 fold) than small luteal cells (Urseley and Leymarie, 1979; Niswender et al., 1985). However, small luteal cells responded to LH stimulation with a large increase (up to 40 fold) in secretion of progesterone, while LH had no effect on large luteal cells (Urseley and Leymarie, 1979, Niswender et al., 1985). During the ovine estrous cycle, luteal 3β-HSD mRNA was expressed maximally by d 3 and remained relatively constant through d 12, while maximum expression of $P450_{scc}$ mRNA did not occur until the midluteal phase (Wiltbank, 1994). This suggests that these enzyme are regulated differentially in ovine luteal cells.

In small luteal cells, LH stimulated-progesterone secretion occurred through activation of phosphokinase A (PKA). This system enhanced cholesterol esterase activity and may enhance transport of cholesterol to the mitochondria (Niswender et al., 1994). In cattle, cholesterol carried by the high density lipoprotein (HDL) is the major substrate for progesterone synthesis for the small and large luteal cells. However, specific HDL receptors have not been identified in luteal cells (Wiltbank, 1994). Activation of PKA in large luteal cells did not stimulate secretion of progesterone (Wiltbank, 1994).

Arachidonic acid has been shown to stimulate progesterone secretion in small and large luteal cells, and its cyclooxygenase metabolites, PGE_2, PGI_2 and $PGF_{2\alpha}$ have been reported to be luteotrophic in small luteal cells (Alila et al., 1988). Small and large luteal cells contain binding sites for PG_{I2}, PG_{E2}: their number increase until mid luteal phase and decline in the late luteal phase (Chegini et al., 1991).

Luteinizing hormone also has an indirect steroidogenic role a by increasing the blood flow and delivery of nutrients to the corpus luteum (Wiltbank, 1994). These observations are reinforced by the identification of binding sites for LH/hCG in luteal blood vessels (Chegini et al., 1991).

Luteolysis

Prostaglandin $F_{2\alpha}$ is the primary luteolytic agent in farm animals. Secretion of $PGF_{2\alpha}$ from uterine origin induces regression of the CL in cattle (for review see Silvia et al., 1991). After administration of $PGF_{2\alpha}$ in ewes, the blood supply to the ovary bearing the CL was decreased and may have cause hypoxia of luteal tissue (Nett et al., 1976). However, it remains unclear if this decline in luteal blood flow is a symptom or a cause of luteolysis.

Receptors for $PGF_{2\alpha}$ are located on small and large luteal cells and $PGF_{2\alpha}$ binding to its receptor increased from early to late luteal phase (Chegini et al., 1991). Antisteroidogenic effects of $PGF_{2\alpha}$ are mediated through a phosphokinase C (PKC) second messenger system and induction of intracellular release of free calcium (Niswender et al., 1994). The release in free calcium may induce apoptosis in large luteal cells (Juengel et al.,1993). The reason for regression of small luteal cells is not known, but secretion of a cytotoxic factor from the large luteal cells has been suggested as well as involvement of immune cells (Pate, 1994).

Factors Affecting CL Function

Gonadotropin Requirements

There has been considerable controversy regarding requirements of the anterior pituitary gland for regulation of CL function in ewes and cows. Denamur et al. (1973) demonstrated that hypophysectomy of hysterectomized ewes resulted in CL regression. In cattle, Donaldson and Hansel (1965b) showed that injection of purified bovine LH in Freund's adjuvant given at mid-cycle prolonged the lifespan of the CL and lengthened the estrous cycle in cattle. LH-containing preparations and crude bovine pituitary extracts significantly increased CL weight and progesterone content in hysterectomized heifers (Hansel and Seifart, 1967). The administration of equine anti-bovine LH serum caused partial regression of corpora lutea in hysterectomized heifers (Hansel and Seifart, 1967). Finally, progesterone synthesis by cultured bovine CL slices is stimulated by bovine-LH. This response to LH is highly sensitive, specific, and inhibited by addition of equine anti-bovine LH serum (Hansel and Seifart, 1967).

Research by Villa-Godoy et al. (1985) suggested that the results obtained by Hansel and Seifart (1967) can be interpreted differently. Prolongation of the CL lifespan obtained by supplementation with LH, may be due to luteinization of the dominant follicle that appears in the last part of the estrous cycle. This follicle is very estrogenic, and estradiol plays an important role in regulating the phenomenon of luteolysis. Destruction of ovarian follicles and inhibition of follicular growth on d 9, d 12 or d 15 after estrus extended the luteal function and lifespan probably by delaying the uterine release of $PGF_{2\alpha}$ (Villa-Godoy et al., 1985). Peters et al. (1994) administered an antagonist of LH releasing hormone (LHRH) and established that LH support was essential to CL development and function from d 2 to d 12 after estrus. However, CL function was not altered by suppression of LH support via treatment with an antagonist of LHRH from d 12 to d 17 after estrus. This result is consistent with the expectation that an abundant population of large luteal cells derived from small luteal cells do not require LH for progesterone secretion (Niswender et al., 1985). Prostaglandins I_2 and E_2 of either uterine or ovarian origin also are required for the normal development of CL, since CL function is altered when inhibitors of prostaglandin synthetase are administered after ovulation (Milvae and Hansel, 1985; Chegini et al., 1991; Hansel et al., 1991).

Dysfunction of the CL

Dysfunction of CL can be categorized into two types: 1) corpora lutea having a short lifespan (Copelin et al., 1987) and 2) corpora lutea having normal lifespan but reduced progesterone secretion (Pratt et al., 1982). Short luteal phases have been documented in sheep and cattle during puberty, following the first spontaneous ovulations post-partum, and at the start of the breeding season in anestrous ewes (reviewed by Lauderdale, 1986). The short estrous cycle contributes to infertility of the first breeding after post-partum anestrus by causing the CL to regress before maternal recognition of pregnancy occurs (Short et al., 1990). Normal length luteal phases can be induced in post-partum cows by pre-treating with exogenous progestins for 6 to 10 days (Ramirez-Godinez et al., 1981). The short-luteal phases have been shown to be due to a premature release of luteolysin from the uterus (Copelin et al., 1987; Zollers et al, 1991).

Premature luteal regression of the CL is the result of early activation of the normal luteolytic mechanism (involving the coincident secretion of oxytocin from the CL and $PGF_{2\alpha}$ from the uterus) as a result of high concentrations of uterine oxytocin receptors during the early luteal phase (Garverick et al., 1992). The absence of progesterone stimulation before estrus increased the number of estradiol and oxytocin receptors in the uterus. The estrogen secreted by the first wave of follicular growth stimulated formation of uterine oxytocin receptors and induced early regression of the CL (Beard and Hunter, 1994). This observation is supported by a decrease in concentrations of progesterone receptors in the early luteal phase in post-partum cattle when progesterone pretreatment is omitted (Zollers et al., 1991). The normal rapid rate of decline in oxytocin receptors after estrus is then diminished under the stimulation of estradiol secreted by the first wave of follicular growth (Garverick et al., 1992; Beard and Hunter, 1994).

An inadequate gonadotropin stimulation of the preovulatory follicle cannot be completely discounted. Differences were reported between preovulatory follicles destined to be normal (treated with a progestin implant) versus short lived corpora lutea (untreated) in cattle. Breuel et al. (1993) treated post-partum suckler beef cows with a norgestomet implant for 9 days and induced estrus by weaning the calves 2 days before removal of the implant. Treated cows had a lower frequency of short estrous cycles and a higher increase of mean concentrations of LH and estradiol during the 3 days preceding estrus when compared to untreated control cows. This difference in LH and estradiol pattern of secretion may result in a better gonadotrophic priming of the pre-ovulatory follicle and improve the synchrony between follicular maturation and the pre-ovulatory surge of LH resulting in the formation of a CL with a normal lifespan. Furthermore, the norgestomet implant induced an increase in pulse frequency of LH on d 6 after insertion (Garcia -Winder et al., 1986) and an increase in LH and FSH receptors of the dominant follicle in norgestomet treated cows compared to control cows (Inskeep et al., 1988; Braden et al., 1989).

In sheep, insufficient luteal phases occur commonly when attempts are made to induce ovulation during the seasonal anestrus with GnRH. A single bolus injection of large amount of GnRH (40 µg) resulted in a discharge of LH, ovulation and formation of a CL. However, plasma concentrations of progesterone remained either basal or rose to a lower level than that observed during normal luteal phases (Haresign et al., 1975; Hunter, 1991). When GnRH administration is modified to a regimen which simulated a progressive increase in LH pulses which normally occurs during the transition from the luteal to the follicular phase of the breeding season, a much higher proportion of ewes ovulated and had luteal phases of normal length with normal plasma progesterone concentrations (McNatty et al., 1981; Baird, 1992).

Development of the CL begins before ovulation as part of follicular maturation since granulosa cells undergo luteinization and begin to secrete progesterone before ovulation (McNatty, 1979). In primates, insufficient gonadotropic stimulation of the follicle during the pre-ovulatory period was associated with insufficient progesterone secretion by the subsequent CL (DiZerega and Hodgen, 1981; Zelinski-Wooten et al., 1991; Jones, 1991). For example, exposure to a pre-ovulatory surge of LH of 10 h is insufficient to initiate final meiotic maturation of oocytes or support the early function of the CL in primates (Zelinski-Wooten et al., 1991). Primates appear to require a pre-ovulatory LH surge of 48 h to initiate normal follicular maturation and CL function. If expression of progesterone receptors in granulosa cells is used as a marker of luteinization, then a pre-ovulatory surge of 48 h is necessary to induce differentiation of granulosa cells (Chandrasekher et al., 1991).

Collectively, these results suggest that the endocrine environment to which the follicle is exposed before ovulation has a fundamental influence on the subsequent function of the CL.

Manipulation of Reproductive Function in Cattle

Estrus Synchronization

Effective estrus detection requires skilled labor and considerable time, and is often cited as a major component in determining the effectiveness of a breeding program (Macmillan, 1992). Barr (1974) reported that increased calving intervals have a correlation of .92 with failure to observe estrus and .38 with failure to conceive. Implementation of estrous synchronization programs is essential for efficient use of artificial insemination and embryo transfer to improve the genetic potential of beef and dairy cattle. Ideally, an estrous synchronization system should elicit a fertile, tightly synchronized estrous response in a high percentage of treated females (Odde, 1990). Three responses (Lauderdale et al., 1981) usually are considered to evaluate synchronization protocols: 1) synchronization rate is the proportion of animals detected in estrus during the 7 days after the end of the synchronization treatment of those entering the synchronization treatment; 2) conception rate is the proportion of animals conceiving of those detected in estrus during the 7 days after the last day of the synchronization treatment; 3) pregnancy rate is the proportion of animals conceiving within 7 days after the end of the synchronization treatment of those entering the synchronization treatment. These definitions are used readily by various investigators, is a nomenclature used by the

Journal of Animal Science and will be adopted for the studies reported in this dissertation.

Several pharmaceutical drugs have been utilized to synchronize estrous cycles estrous cycles and all of them are hormonal in nature (reviewed by Chenault, 1992). Most of the synchronization protocols available manage plasma concentrations of progesterone with luteolytic agents or extended progestogen treatments (reviewed by Odde, 1990). Estrus is the consequence of the coordinated growth of an ovarian follicle, and increased follicular estradiol secretion is coupled with the demise of the CL (Chenault et al., 1975; 1976). Thus, regulation of follicular growth and estradiol secretion combined with regulation of luteal progesterone secretion may provide a means to enhance precision of estrus synchronization and optimize fertility (Macmillan and Henderson, 1984; Fogwell et al., 1986; Thatcher et al., 1989; Kastelic et al., 1990a; Savio et al., 1990c; Macmillan and Peterson, 1993). A recapitulation of the respective performances of the different synchronization treatments is presented in Table 2. However, caution should be exerted in interpreting this information since no direct comparison among different treatments is performed in a single experiment.

Table 2. Summary of selected references on the performances of various synchronization protocols in heifers

Treatment	No. of animals	Estrus rate, % (5 to 7 days after treatment)	Conception rate, % (5 to 7 days after treatment)	Pregnancy rates,% (5 to 7 days after treatment)
GnRH-day 0 $PGF_{2\alpha}$-day 7 [1]	355	80	63	50
Syncro-Mate B [2]	566	92	46	42
$PGF_{2\alpha}$ -2 injections 10 to 11 days apart [3]	462	66	55	38
7 days CIDR + $PGF_{2\alpha}$ [4]	234	95	60	57
estradiol benzoate + 12 days CIDR [4]	443	94	56	53
14 days-MGA $PGF_{2\alpha}$ 16 to 18 days after MGA [5]	157	83	69	57

[1] Schmitt et al. (1994)
[2] Odde (1990)
[3] Lauderdale et al. (1981)
[4] Macmillan and Henderson (1993)
[5] Brown et al. (1988)

Synchronization of Estrus with Progesterone or Progestins

Progestogens suppress estrus in cattle and have been administered in different ways and treatment combinations to alter the estrous cycle (Odde, 1990).

Melengestrol acetate (MGA). Melengestrol acetate is an orally active synthetic progestin that suppresses estrus and ovulation in treated cattle. Zimbelman et al. (1970) reviewed 24 studies that addressed the effectiveness of MGA fed for 10 to 18 days as an estrous synchronization agent. The percentage of MGA treated females in estrus in a 6-d period following treatment was similar to the percentage of controls in estrus in a 20-d period. However, conception rate was

14 % lower for MGA treated cattle than for controls. Recently several investigators established that the reduced fertility after a prolonged MGA treatment was due to the formation of persistent follicles. Custer et al. (1994) reported that a 7-d MGA treatment initiated on d 17 of the estrous cycle altered the normal course of ovarian follicular dynamics by maintaining growth and development of the follicle that was dominant at the beginning of the MGA treatment. Maintained dominance was reported by others and resulted in reduced conception rates upon ovulation of the maintained dominant follicle (Savio et al., 1993b). Melengestrol acetate, fed at greater doses than typically used for estrous synchrony in cattle does not mimic endogenous progesterone produced by the CL in regulation of secretion of LH and estradiol-17β (Kojima et al., 1995a). Therefore, in the absence of a CL, cattle fed MGA present a pattern of high frequency LH pulsatility characteristic of the pattern observed during the follicular phase of the estrous cycle that maintains growth of the dominant follicle (Custer et al., 1994; Kojima et al., 1995a) and results in lowered conception rates. Thus artificial progestins do not have necessarily the same physiological effects as progesterone and should be used cautiously (Kojima et al., 1992).

Different strategies for the use of MGA treatment in combination with $PGF_{2\alpha}$ were developed for synchronization of estrus in cattle (Brown et al., 1988; Patterson et al., 1989; Odde, 1990; Chenault et al., 1990b; Coleman et al., 1990). Brown et al. (1988) proposed a synchronization protocol with MGA fed for 14 days and $PGF_{2\alpha}$ administered 16 to 18 days after progesterone withdrawal. Such a treatment placed the animals in the luteal stage of the estrous cycle at the time of the luteolytic injection and resulted in good synchronization and fertility at estrus when compared to cows treated with Syncro-Mate B (Table 2).

Progesterone and progesterone releasing devices. Daily injection of progesterone was described as a means to synchronize estrus in cattle (Ulberg et al., 1951; Wiltbank et al., 1965). Progesterone releasing devices (intravaginal releasing device) allow continuous delivery of progesterone and eliminate the necessity of daily injections (Roche, 1974b; Sreenan, 1975; Macmillan and Peterson, 1993). Like MGA feeding, 14 to 21 days of progesterone administration results in excellent synchronization rates but conception rates are reduced (Roche, 1974; Macmillan and Peterson, 1993). However, progesterone treatment for shorter periods of time without the use of a luteolytic agent does not result in acceptable synchronization rates because progesterone from the device has variable effects on CL lifespan according to the day of treatment. Treatment with progesterone during the early part of the estrous cycle reduces CL lifespan (Macmillan and Peterson, 1993), lowered progesterone synthesis by the corpus luteum and increased its sensitivity to $PGF_{2\alpha}$ (Herring and Lauderdale, 1980). When a Control Internal Drug Releasing (CIDR-B) device was inserted on d 1 after estrus for 3 or 4, days the inter-estrus interval was reduced to an average of 8 and 10 days, respectively. However, device insertion at mid-cycle (d 9 to d 11) did not alter plasma progesterone concentration or length of the estrous cycle (Macmillan and Peterson, 1993, Burke et al., 1994). Therefore, protocols for estrus synchronization that incorporate progesterone releasing devices for a reduced period of time need to involve the use of luteolytic agents (estrogens or $PGF_{2\alpha}$).

Progestin/progesterone releasing devices combined with estrogen. Estrogens were reported to be luteolytic when given during the early part of the estrous cycle (Wiltbank et al., 1961). Administration of estradiol on d 13 of the estrous cycle in heifers induced an acute increase of circulating $PGF_{2\alpha}$ metabolite (PGFM) and hastened occurrence of estrus when compared to heifers treated with a placebo (Thatcher et al., 1986). Wishart and Young (1974) administered estrogen as an intramuscular (i.m.) injection at the start of a 9-d progestogen implant treatment. The estrogen was to serve as a luteolytic agent and the progestin implant was to inhibit development of CL in heifers that had recently ovulated or prevented ovulation if the treatment was to be initiated in the last part of the estrous cycle. Wiltbank and Gonzales-Padilla (1975) reported that a 9-d treatment with an implant containing 6 mg norgestomet and an injection of estradiol valerate (5 mg) plus of norgestomet (3 mg) given at the time of implant insertion, successfully synchronized estrus. This treatment is available commercially as Syncro-Mate B. Synchronization rates after treatment with Syncro-Mate B are high with 77% to 100% of treated animals showing estrus in most trials (reviewed by Odde, 1990). However, conception rates to the Syncro-Mate B treatment is influenced by the day of the estrous cycle when treatment is initiated (Odde, 1990). More importantly, it appears that the estradiol valerate injected during the Syncro-Mate B treatment also affects follicular dynamics (Adams, 1994; Bo et al., 1995).

Ultrasonographic examinations revealed that estradiol valerate treatment at the time of the norgestomet implant insertion (d 2 after estrus) was associated with a reduction in the mean diameter of the largest and second largest follicles over a 5-d period followed by the recruitment of a new wave of follicular growth (Bo et al., 1995). Estradiol benzoate injected at the time of treatment with a progestogen implant during the period of growth, dominance or regression of the first wave dominant follicle induced regression of the dominant follicle and emergence of a new follicular wave 4.3 ± .6 days after initiation of treatment (Adams, 1994). Therefore, the estradiol valerate / norgestomet injection given at the initiation of the Syncro-Mate B treatment may also synchronize follicular growth. This may explain the results of Witthier et al. (1986) who reported an increased precision of estrus when the combination of a norgestomet implant / estradiol benzoate injection at implant insertion was compared to a treatment with a norgestomet implant and an injection of $PGF_{2\alpha}$ at the time of implant removal. The variability of the results observed with Syncro-Mate B treatments (Odde, 1990) may be due to the inconsistent effect of the estradiol valerate on the dominant follicle that may attributed to the long half life of the estradiol valerate (Bo et al., 1995). The use of estradiol benzoate in place of estradiol valerate may alleviate this problem (Bo et al., 1995). Treatment of ovariectomized heifers with the Syncro-Mate B protocol demonstrated that the induced estrus after treatment with Syncro-Mate B was due to the residual estradiol from the administration of estradiol valerate. More than 50 % of the ovariectomized heifers treated with Syncro-Mate B exhibited estrus and had an LH surge within 72 h after the implant removal. The residual estradiol in serum after the Syncro-Mate B in ovariectomized heifers that exhibited estrus was at a concentration known to induce estrus in ovary-intact heifers (Larson and Kiracofe, 1995). Perhaps the reduced or variable fertility associated with estrous periods after Syncro-Mate B treatment (Brink and Kiracofe, 1988) is due to anovulatory estrus or improper timing of insemination relative to follicular maturation and ovulation.

Two other progesterone releasing devices are commercially available for estrus synchronization in certain parts of the world: CIDR-B device and the Progesterone Releasing Intravaginal Device (PRID; Chenault, 1992). A gelatin capsule of oestradiol benzoate (10 mg) can be inserted into the vagina in combination with the CIDR-B device at initiation of the treatment sequence to induce luteolysis and/or recruitment of a new follicular wave (Macmillan and Peterson, 1993).

Progestin/progesterone releasing devices combined with $PGF_{2\alpha}$. The use of $PGF_{2\alpha}$ with progesterone or progestin releasing devices has been proposed at the time of implant removal (Smith et al., 1984; Odde, 1990; Macmillan and Peterson, 1993) or 1 to 2 days before removal of the implant (Smith et al., 1984; Macmillan and Peterson, 1993). The progesterone implant is kept in place 7 to 9 days. Injection of prostaglandin 24 h or 48 h before removal of the implant has been suggested as a means to enhance precision of estrus and allow timed-insemination (Smith et al., 1984; Macmillan and Peterson, 1993). It is possible that the increased LH pulsatility during the period between luteolysis due to the administration of $PGF_{2\alpha}$ and removal of the progesterone/progestin implant, permits a more homogenous growth of the pre-ovulatory follicle. Furthermore, $PGF_{2\alpha}$-induced regression of the CL permits a more consistent withdrawal from progesterone with removal of the implant. These factors appear to enhance precision of estrus.

Synchronization of Estrus with $PGF_{2\alpha}$

Prostaglandin $F_{2\alpha}$ and its analogs are probably the most used compound for estrous cycle control in cattle. Its properties on the estrous cycle are well established and have been described in numerous reviews (Inskeep, 1973; Thatcher and Chenault, 1976). Prostaglandin $F_{2\alpha}$ induces regression of the CL, followed by an increase in LH pulse frequency inducing maturation and ovulation of the dominant follicle. During the 5 days following estrus, $PGF_{2\alpha}$ is ineffective in inducing luteolysis (Lauderdale, 1972). Since high affinity $PGF_{2\alpha}$ receptors are present in the CL by d 2 after ovulation, this lack of responsiveness of the CL is probably due to a defect in coupling of the $PGF_{2\alpha}$ receptors to intracellular second messengers or to an incomplete differentiation of the degenerative mechanisms in newly differentiated luteal cells (Wiltbank et al., 1995). Several management systems based on the use of $PGF_{2\alpha}$ have been described (reviewed by Lauderdale et al., 1981; Odde, 1990; Chenault, 1992).

One limitation of the $PGF_{2\alpha}$ synchronization programs is the precision of estrus synchrony. For example, response to $PGF_{2\alpha}$ in terms of degree of synchrony will vary with the day of estrous cycle (Tanabe and Hann, 1984; Macmillan and Henderson, 1984). Tanabe and Hann (1984) reported that after an administration of $PGF_{2\alpha}$ on d 7, d 9 or d 15 of the cycle, the synchronized estrus occurred at 43.9 ± 8.2 h, 71.5 ± 14.3 h and 53.0 ± 12.2 h, respectively. This variation is probably due to the developmental stage of the dominant follicle when the $PGF_{2\alpha}$ treatment is administered (Macmillan and Henderson, 1984; Savio et al., 1990a; Kastelic et al., 1990a). Injection on d 7 allowed rapid maturation and ovulation of the "healthy" and estrogenic first wave dominant follicle whereas on d 11, several days may be necessary for the selection and maturation of the new second wave dominant follicle resulting in a longer interval from treatment to ovulation. This problem cannot be completely eliminated by giving two injections of $PGF_{2\alpha}$ 11 (heifers) or 14 days (lactating cows) apart (Odde, 1990; Chenault, 1992). Follicular growth is not synchronized with these treatments which were designed to ensure that all the animals will have a fully functional and responsive CL at the second injection of $PGF_{2\alpha}$. Animals at the time of the second injection of $PGF_{2\alpha}$ (in the case of two injections given 11 days apart) will be between d 8 and d 16 of the estrous cycle. Therefore, some animals will ovulate the first wave dominant follicle after the second injection of $PGF_{2\alpha}$ whereas others will ovulate a second or third wave dominant follicle. When injections of $PGF_{2\alpha}$ are given 14 days apart most of the animals will be between d 11 and 19 of the estrous cycle at the time of the second injection and will ovulate a second or third wave dominant follicle.

Fertility at an estrus induced with administration of $PGF_{2\alpha}$ is good (for review see Odde, 1990). However, conception rates may be higher following late luteal phase injection compared with early luteal phase injections (Watts and Fuquay, 1985). Stevenson et al. (1984) did not detect any effect of the stage of the estrous cycle at which $PGF_{2\alpha}$ was administered on fertility. Ferguson and Galligan (1993) reported that synchronization and number of estruses are increased when injections are given 14 days apart when compared to two injections given 11 days apart. Folman et al. (1990) observed that cows given two injections of $PGF_{2\alpha}$ 14 days apart conceived at a higher rate than when the injections were given 11 days apart. Recommended management of reproduction in post-partum dairy cows involves two injections of $PGF_{2\alpha}$ administered 14 days apart with this treatment being initiated after a voluntary waiting period of at least 50 days (Ferguson and Galligan, 1993). Increased plasma concentrations of progesterone before the second administration of $PGF_{2\alpha}$ was associated with an increase in conception rates (Folman et al., 1990). Perhaps repetitive use of $PGF_{2\alpha}$ leading to extended periods of low plasma concentrations of progesterone, may induce low conception rates.

In summary, both progesterone and $PGF_{2\alpha}$ are very efficient tools to induce estrus in cattle, however they could probably be improved if coupled with a system to also regulate waves of follicular growth.

Synchronization of Follicular Waves

Progesterone releasing devices or luteolytic agents mainly focus on abbreviating or lengthening the luteal phase of the estrous cycle. Since estrus is the combination of CL demise and the coordinated growth of an estrogenic follicle (Chenault et al., 1975, 1976; Thatcher and Chenault, 1976), a system that coordinates both follicular growth and CL regression should allow a more precisely synchronized estrus with good fertility.

Synchronization of follicular growth with progesterone. Synchronization of estrus with progesterone/progestin for an extended period of time (18 to 21 days) does indeed synchronize follicle development. During this extended period, the CL will regress and the dominant follicle will be maintained in a low progestin environment and primed with a high basal secretion of LH that leads to a precise synchronization of estrus (Macmillan and Peterson, 1993). However, as mentioned previously, fertility upon ovulation was reduced. Some effects of acute progesterone exposure on follicular growth and follicle development have been examined recently (Adams et al., 1992a; Wehrman et al., 1993; Custer et al., 1994, Anderson and Day, 1994). Progesterone or progestins given in sufficient amounts suppressed LH support of the dominant follicle and induced atresia. This loss of dominance was followed by an increase in FSH secretion and recruitment of a new follicular wave (Adams et al., 1992a). Therefore, by inducing regression of the dominant follicle, progesterone can trigger recruitment of a new follicular wave.

Synchronization of follicular waves with estradiol/estrogens. As discussed previously, estradiol valerate, given in combination with a progestogen can alter the pattern of follicular growth and induce recruitment of a new follicular wave (Adams, 1994; Bo et al., 1995). This is probably the major effect of the estrogens used with the Syncro-Mate B, CIDR-B and PRID treatments. It is interesting to note that the proposed luteolytic effect of the estradiol treatment is probably of minor importance and is more likely acting to recruit a new wave of follicular growth. However, the hypothesis that follicular wave emergence can be elicited with an injection of estradiol associated with a progestin implant treatment in a synchronous manner within a group of randomly cycling cows (Bo et al., 1995) has never been tested directly.

Synchronization of follicular growth by follicular ablation. A direct approach to eliminate the dominant follicle's suppression of the next wave of follicular growth is direct ablation of the dominant follicle (Adams et al., 1992a; Wolfsdorf et al., 1994) by electrocauterization or transvaginal aspiration of the dominant follicle guided by ultrasonography. Both techniques induce elimination of the dominant follicle and reduce inhibin secretion. This alteration in follicle function allows a surge of FSH to occur and recruitment of a new follicular wave. However, this method is very cumbersome and of little practical value for management of estrus synchronization on a large scale.

Synchronization of follicular growth with GnRH or GnRH-agonist. Administration of a potent GnRH-agonist on d 11, 12 or 13 of the estrous cycle

(Buserelin, 10 µg, i.m.) induces ovulation or luteinization of large and medium size follicles present on the ovary at the time of treatment. This is followed by recruitment of a new wave of follicular growth (Thatcher et al., 1989; Macmillan and Thatcher, 1991). Similar results were obtained by Twagiramungu et al. (1994a,b) when the animals were treated with an injection of 8 µg of Buserelin on d 7 of the estrous cycle. When Buserelin was administered at a random time during the estrous cycle, occurrence of estrus during the 7 days following treatment was reduced when compared to animals that did not receive any treatment (Thatcher et al., 1989). These results confirm that a GnRH-agonist can eliminate a dominant follicle and recruit a new follicular wave. An injection of GnRH or GnRH-agonist induces a surge of LH and FSH (Chenault et al., 1990a). The LH release is sufficient to induce ovulation or luteinization of the large follicles present on the ovary at the time of treatment (Macmillan and Thatcher, 1991; Twagiramungu et al., 1994a; Wolfenson et al., 1994). Elimination of the dominant follicle by the GnRH-induced surge of LH reduces inhibin secretion and allows for an endogenous surge of FSH that supplements the GnRH-induced surge of FSH to induce recruitment of a new wave of follicular growth. Therefore, GnRH efficiently induces recruitment of a new follicular wave in cattle and has been tested for this purpose in estrous synchronization programs (Thatcher et al., 1993; Twagiramungu et al., 1995a). Synchronization with administration of a GnRH-agonist followed 7 days later by an injection of $PGF_{2\alpha}$ results in conception rates that do not differ from what is obtained after two injection of $PGF_{2\alpha}$ given 11 days apart (Coleman et al., 1991).

Synchronization of Ovulation / Timed Insemination

Existing estrous synchronization protocols could be further improved by synchronizing the time of ovulation and therefore eliminating the need for estrus detection. Different approaches have been taken to allow insemination at a fixed time after synchronization treatment.

Lauderdale et al. (1981) proposed a synchronization system where beef cows or heifers received two consecutive injections of $PGF_{2\alpha}$ and were inseminated 77 to 80 h after the last injection without any detection of estrus. After this treatment, pregnancy rates did not differ when compared to animals inseminated at detected estrus after synchronization with 2 injections of $PGF_{2\alpha}$ (34% versus 35%, for cows; 38 versus 36%, for heifers). However Lucy et al. (1986) reported that in Holstein cows treated with two injections of $PGF_{2\alpha}$ given 11 days apart and inseminated at 80 h after the last injection, conception rates were lower (23%) than the control group (51%, insemination at estrus occurring naturally). No comparison to an appropriate control (two injections of $PGF_{2\alpha}$ and insemination at observed estrus) were made in this last experiment. Some variation in the results observed from one experiment to another could be attributed to variability in the time of estrus observed after synchronization of estrus with $PGF_{2\alpha}$ (Macmillan and Henderson, 1984; Tanabe and Hann, 1984, Smith et al., 1984).

Therefore a major factor for consideration in developing a timed insemination program after $PGF_{2\alpha}$ treatment is whether heats after treatment are grouped sufficiently to allow for a timed single insemination. An additional hormonal treatment after $PGF_{2\alpha}$ injection may be useful to narrow the window of ovulation and increase the efficacy of a timed-insemination.

Thatcher and Chenault (1976) proposed to induce ovulation after synchronization with $PGF_{2\alpha}$ with an administration of GnRH. They demonstrated that injection of GnRH given 24 or 48 h after $PGF_{2\alpha}$ would elicit a release of LH. However, fertility to a timed-insemination made 15 h after an injection of GnRH that was given 48 h after a single administration of $PGF_{2\alpha}$ tended to be reduced when compared to $PGF_{2\alpha}$ alone and insemination at detected estrus (Thatcher and Chenault, 1976). This difference was attributed to a GnRH treatment administered too prematurely to achieve good fertility.

Peters et al. (1977), proposed to induce estrus in cattle with a palpable CL, with a single administration of $PGF_{2\alpha}$ followed by an injection of 400 µg of estradiol benzoate at 48 h. Insemination was performed at 72 or 96 h after the last injection of $PGF_{2\alpha}$. This treatment improved the precision and expression of estrus with no alteration of conception rates when compared to animals treated with $PGF_{2\alpha}$ only.

Roche (1975) proposed a program to control the time of ovulation in heifers based on treatment with a progesterone releasing device for 11 days with an injection of 5 mg of estradiol benzoate and 50 mg of progesterone given at the time device was inserted. Thirty hours after removal of the coil an injection of GnRH was given. This treatment induced ovulation in 8 of 12 heifers by 60 h after removal of the implant, however, no pregnancy data were available.

These protocols to control the time of ovulation to permit insemination at a fixed time were never used on a wide scale, probably due to important variations in the results.

In summary, several hormones can be used effectively to induce luteolysis, follicular recruitment and ovulation in cattle. However, regulating CL function, follicular growth and ovulation in an integrated treatment sequence may lead to new therapies for estrous synchronization that allow appointment breeding. To achieve this goal, studies that monitor closely the effect of hormonal treatments on the ovariaæn and uterine physiology are necessary.

CHAPTER 3

ELIMINATION OF PERSISTENT FOLLICLES AND RECRUITMENT OF NEW DOMINANT FOLLICLES WITH A GONADOTROPIN-RELEASING HORMONE AGONIST IN CATTLE: EFFECT ON CONCEPTION RATES

Introduction

During the luteal phases of estrous cycles in heifers and cows, two to three waves of follicular development occur. Each wave is characterized by development of a follicle that undergoes stages of recruitment, selection, and dominance (Savio et al., 1988; Ginther et al., 1989; Lucy et al., 1992). During the period of dominance, the follicle can either undergo atresia, ovulate, or persist as an estrogenic dominant follicle depending on the hormonal milieu (e.g., progesterone and LH concentrations).

Turnover of the dominant follicle is regulated by plasma concentrations of progesterone via a negative feedback on LH secretion. A low concentration of plasma progesterone increased the frequency of LH pulsatility and stimulated growth of the dominant follicle, whereas a high concentration of plasma progesterone decreased LH pulse frequency leading to turnover of the dominant follicle in cattle (Roberson et al., 1989; Savio et al., 1990, 1993a). The first-wave dominant follicle was programed to continue development and not undergo turnover when the corpus luteum (CL) was regressed on d 7 of the estrous cycle with a $PGF_{2\alpha}$ injection. Continued follicle development was achieved under a low progestogen environment with administration of a norgestomet implant or progesterone via an intravaginal device (Cupp et al., 1993; Savio et al., 1993a,b). This persistent follicle was highly estrogenic and dominant. It ovulated after implant removal (Savio et al., 1993a,b). However, fertility was reduced after insemination at the estrus induced by a persistent follicle (Savio et al., 1993b). Several studies indicated that injection of an agonist of GnRH (Buserelin, 8 µg, i.m.) synchronized development of a new follicular wave after induced ovulation or luteinization of follicles $\geq$ 9 mm (Macmillan and Thatcher, 1991; Stevenson et al., 1993; Wolfenson et al., 1994; Twagiramungu et al., 1994). The newly recruited follicle induced estrus when $PGF_{2\alpha}$ was administered 7 d after injection of the GnRH-agonist. Furthermore, fertility at the induced estrus was not reduced (Thatcher et al., 1989; Coleman et al., 1991; Twagiramungu et al., 1992a,b).

Objectives of the present experiments were to determine whether an injection of GnRH-agonist would ovulate a persistent first wave dominant follicle and induce recruitment of a new dominant follicle with improved fertility.

Materials and Methods

Experiment 1

An initial study was conducted at the University of Florida Dairy Research Unit (Hague) to determine whether injection of GnRH-agonist would ovulate the first wave dominant follicle and recruit a new dominant follicle that might ovulate after withdrawal of a progestin implant (Figure 3-1). Previous studies (Savio et al., 1993a) indicated that dominance of the first-wave dominant follicle is extended beyond d 18 when maintained in a low progestin environment. Therefore, the same model (Savio et al., 1993a) to induce a potentially persistent first-wave dominant follicle was used.

Nonlactating Holstein cows (n = 10) were kept in a lot with minimal pasture and fed peanut hay and corn silage. Estrus was synchronized with s.c. insertion of a norgestomet ear-implant (Syncro-Mate B; 6 mg of norgestomet implant used without the injection of the norgestomet/estradiol valerate solution; Sanofi Animal Health, Overland Park, KS) for 7 d and an injection of $PGF_{2\alpha}$ (Lutalyse ; 25 mg i.m.; The Upjohn Company, Kalamazoo, MI) 24 h before implant removal. Cows were observed for estrus twice daily (0600 to 0700 and 1800 to 1900) after removal of the implant. Tail heads were painted (Impervo, Benjamin Moore and Co., Montvalo, NJ) and chalked (All-weather Paintstick, LA-CO Industries, Chicago, IL) as an aid for estrus detection (Macmillan et al., 1988). Five cows observed in estrus on the same day (d 0) entered the following treatment sequence. On d 7, cows received a norgestomet ear-implant and a $PGF_{2\alpha}$ (Lutalyse, 25 mg i.m.) injection. This treatment sequence induced a low progestin environment, increased the frequency of LH pulsatility, and stimulated continuous growth of the first-wave dominant follicle (Taylor et al., 1993; Savio et al., 1993a). On d 9, all cows received an injection of a GnRH-agonist (Buserelin, 8 µg i.m.; Hoechst-Roussel Agri-Vet, Somerville, NJ) to ovulate the first-wave dominant follicle and induce recruitment of a new follicular wave (Thatcher et al., 1989; Macmillan and Thatcher, 1991). Norgestomet implants

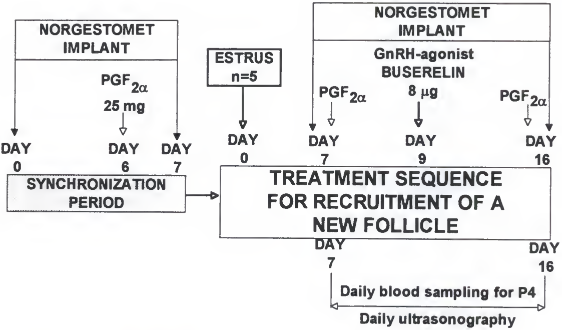


Figure 3-1. Treatment sequence for recruitment of a new follicle with a GnRH-agonist injection in a low progestin environment.

were removed on d 16 and cows received a second injection of $PGF_{2\alpha}$ to regress any corpora lutea that might have been induced by the GnRH-agonist. After withdrawal of the norgestomet implant, estrus detection was performed as described previously.

Daily from d 7 to ovulation after implant removal, ovaries in each cow were examined transrectally with an Equisonic LS 1000 linear-array ultrasound device equipped with a 7.5 MHz transducer (Tokyo Keiki Co., Tokyo, Japan). Ovarian structures (follicles and corpora lutea) were measured, and their relative positions were recorded on follicular maps drawn during the examination.

Blood (10 ml) was collected daily before to each ultrasonographic examination. Samples were collected by jugular venipuncture into evacuated heparinized tubes (Vacutainer®, Becton Dickinson, Rutherford, NJ) and immediately centrifuged (3,000 x *g* for 20 min). Plasma was separated and stored at -20°C until it was assayed for progesterone (P4; Knickerbocker et al., 1986) and estradiol (E_2; Badinga et al., 1992). Plasma concentrations of E2 and P4 were measured with single-antibody RIA. Sensitivity of the E2 assay was .65 pg/ml. Intraassay CV was 8.2%. The minimum detectable concentration of P4 was .3 ng/ml. Intraassay CV was 7.2%.

Experiment 2

Objective of this experiment was to compare fertility from AI at an estrus induced by a newly recruited follicle (GnRH-agonist induced) with that of an estrus induced by a 16-d-old persistent first-wave dominant follicle. The experiment was conducted at a heifer unit in a large commercial dairy (Larson Dairy, Okeechobee, FL). Estrus in a group of 212 heifers was synchronized with a single $PGF_{2\alpha}$ injection (Lutalyse, 25 mg i.m.). Heifers were observed twice daily for 90 min for detection of estrus (0600 and 1800). A modified tail head paint and chalk system

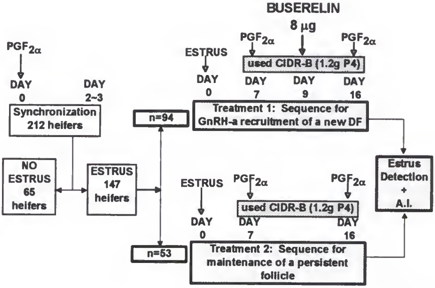


Figure 3-2. Experimental design of Experiment 2. Heifers were treated with a used Controlled Internal Drug Releasing Device (CIDR-B) containing 1.2 g of progesterone (P4) to induce maintenance of a persistent first wave dominant follicle (DF). Heifers in treatment 1 received an injection of GnRH-agonist (Buserelin) to eliminate the persistent DF and recruit a new follicular wave.

was implemented to aid in detection of estrus (Macmillan et al., 1988). Heifers observed in estrus were assigned disproportionally, but randomly, to two treatments (T1 = GnRH-agonist and T2 = Control; Figure3-2). The basic scheme of the experiment was to induce a potentially persistent first-wave dominant follicle. On d 7, all heifers (GnRH-agonist and Control) received an injection of $PGF_{2\alpha}$ (Lutalyse, 25 mg i.m.) and a used CIDR-B device (Controlled Internal Drug Release-Bovine, Eazi-breed, AHI Plastic, Hamilton, New Zealand) to induce subluteal plasma concentrations of progesterone ($2.9 \pm .3$ ng/ml; Savio et al., 1993b) and maintain growth of the first-wave dominant follicle. The CIDR-B devices, used for 9 d in an unrelated experiment, were cleaned, gas-sterilized, and inserted vaginally into all experimental heifers. Because the experiment was conducted in a commercial dairy and pregnancy rate in the group with a persistent first-wave dominant follicle was expected to be reduced (Savio et al., 1993b), heifers were assigned in a manner (GnRH-agonist > Control) that would minimize decreases in herd reproductive efficiency. For Treatment 1, a GnRH-agonist was injected on experimental d 9 to ovulate the potentially persistent first-wave dominant follicle and to recruit a new dominant follicle as described in Experiment 1. A second injection of $PGF_{2\alpha}$ was given at the time of CIDR-B removal on d 16 to regress corpora lutea (CL) induced by injection of GnRH-agonist on d 9 (Figure 3-3). The experimental sequence of Treatment 2 was identical to Treatment 1 except that no GnRH-agonist injection was given. This allowed continuous growth of the first-wave dominant follicle in a low progestin environment until $PGF_{2\alpha}$ injection and

CIDR-B removal on d 16. The persistent first-wave dominant follicle was expected to ovulate (Savio et al., 1993b).

Heifers subsequently observed in estrus for both treatments were inseminated by a single technician using semen of good fertility from eight bulls. Pregnancy diagnosis was performed by palpation per rectum at 45 d after AI.

For the second experiment, a different source of progestin was used to maintain a low level of progesterone. Syncro-Mate B implants were used in the first experiment because norgestomet does not cross-react with the antibody used in P4 RIA (unpublished data). This allowed us to monitor the P4 produced by the regressing CL at d 7 after injection of $PGF_{2\alpha}$ and P4 produced by theGnRH-agonist induced corpus luteum. The CIDR-B devices induced, in the absence of a corpus luteum, the low plasma concentrations of progesterone necessary for maintenance of a persistent follicle in heifers (Savio et al., 1993b). Furthermore, the CIDR-B devices were more convenient to use for a field experiment. Two heifers lost their CIDR-B device during the experimental sequence and were removed from the experiment.

Statistical Analyses

Synchronization rate was defined as the number of animals showing signs of estrus during the 7 d after injection of $PGF_{2\alpha}$ and withdrawal of the CIDR-B device per number of animals receiving $PGF_{2\alpha}$ and CIDR-B devices. Pregnancy rate was defined as the number of heifers pregnant at 45 d after AI divided by the number of heifers assigned to a treatment. Conception rate was defined as the number of heifers pregnant at 45 d after AI divided by the number of heifers inseminated at a detected estrus during the 7 d after the $PGF_{2\alpha}$ injection, for each treatment. Analyses of the data were performed with method of least-squares ANOVA using the GLM procedures, and the CATMOD procedures for analysis of categorical data (SAS, 1988). Mathematical models for statistical analysis included effects of treatment, bull, and the interaction of treatment x bull.

Results

Experiment 1

All cows (n = 5) had a first-wave dominant follicle and a corpus luteum on d 7 of the estrous cycle. Plasma concentrations of progesterone decreased abruptly after injection of $PGF_{2\alpha}$ on d 7 (Figure 3- 3). The decrease in plasma progesterone from d 7 (P4 = 10.4 ± .3) to d 10 (P4 = .9 ± .3 ng/ml) was associated with a decrease in size of the corpus luteum (CL1). Regression of the corpus luteum (CL1), as assessed with ultrasonography (luteal structure no longer visible), was completed by d 10 after estrus. At this time, average plasma concentration of progesterone was < 1 ng/ml (Figure 3-3). Growth of the first-wave dominant follicle (DF1) from d 7 to d 9 was 1.5 ± .5 mm/d in the low progestin environment maintained by the norgestomet implant. This follicle was highly estrogenic because plasma concentrations of estradiol increased until the injection of GnRH-agonist on d 9 (E2 = 13.1 ± .8 pg/ml; Figure 3-3). Injection of GnRH-agonist induced ovulation

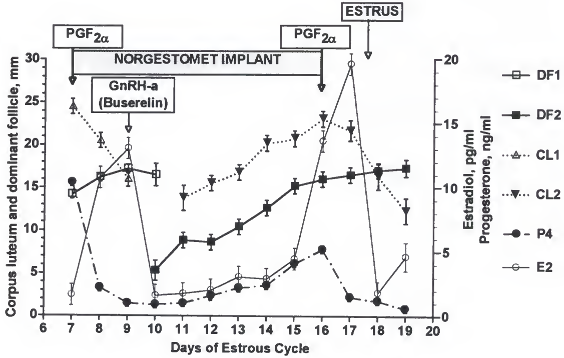


Figure 3-3. Least squares means and S.E. for diameter of the original corpus luteum (CL1) and first-wave dominant follicle (DF1), and induced corpus luteum (CL2) and dominant follicle (DF2) after injection of GnRH-agonist. Least squares means and S.E. for plasma concentrations of progesterone (P4) and estradiol (E2) are presented to characterize functional ovarian changes throughout the experimental sequence.

of the first-wave dominant follicle (DF1) in all cows (5/5). Plasma concentrations of progesterone subsequently increased from d 12 (P4 = 1.6 ± .3 ng/ml) to d 16 (P4 = 5.2 ± .3 ng/ml) in association with appearance and growth of a new corpus luteum following ovulation of the potentially persistent first-wave dominant follicle (DF1) on d 11. The induced corpus luteum (CL2) continued to grow until d 16 when $PGF_{2\alpha}$ was injected (Figure 3-3). The injection of GnRH-agonist on d 9 induced recruitment of a new follicular wave with emergence of a new dominant follicle (DF2) on d 10.4 ± .2. The DF2 became dominant on d 12 ± 1, when its size was 2 mm greater than other follicles recruited in the same wave (Sirois and Fortune, 1990). After the second injection of $PGF_{2\alpha}$ and withdrawal of the norgestomet implant on d 16, plasma concentrations of progesterone decreased in association with a reduction in size of the GnRH-agonist induced corpus luteum (CL2). All GnRH-agonist recruited DF2 produced substantial amounts of estradiol on d 17 (E2 = 19.6 ± .83 pg/ml) before ovulation on d 19.8 ± .2.

Experiment 2

Numbers of heifers in estrus during the 7 d after CIDR-B removal and $PGF_{2\alpha}$ injection did not differ between the two groups (T1 = 96.8% [91/94]; T2 = 94.3% [50/53]; $P < .48$). However, the distribution in days for occurrence of estrus differed between the two groups (Table 3-1; $P < .001$). A greater percentage of heifers (84%) expressed estrus by 48 h after injection of $PGF_{2\alpha}$ and CIDR-B removal in T1. Conception and pregnancy rates differed between treatments (Table 3-2), but no effect of service sire was detected ($P < .64$). Heifers receiving an injection of a GnRH-agonist to induce recruitment and selection of a new dominant follicle for ovulation had greater ($P < .05$) pregnancy and conception rates than heifers ovulating a 16-d-old persistent first-wave dominant follicle. The intervals between first and second estrus in heifers that did not conceive did not differ between treatments.

Table 3-1. Heifers detected in estrus during the 7 days after CIDR-B implant removal and $PGF_{2\alpha}$ injection on day 16 of the treatment sequence[a]

Treatment	Day 1	 2	 3	 4	 5-7	 Total
	percentage (number)					
GnRH-agonist induced ovulatory follicle	1 (1)	84.0 (79)	9.6 (9)	2.2 (2)	0	96.8 (91)
Persistent first-wave ovulatory follicle	0	62.3 (33)	20.7 (11)	11.3 (6)	0	94.3 (50)

[a] $P < .001$ for treatment x day interactions in distribution of estruses (2 x 5 factorial).

Table 3-2. Pregnancy and conception rates at first service for Holstein heifers that potentially ovulated an 8-d-old GnRH-agonist recruited first-wave dominant follicle or a 16-d-old persistent first-wave dominant follicle

Response	GnRH-agonist induced ovulatory follicle	Persistent first-wave ovulatory follicle
Heifers detected in estrus after CIDR-B removal, %	96.8% (91/94)	94.3% (50/53)
Conception rate, % (proportion) [a]	62.6% (57/91)	46% (23/50)
Pregnancy rate, % (proportion) [a]	60.6% (57/94)	43.4% (23/53)
Interval from first to second service, d	23.6 ± 1.3	23.4 ± 1.5

[a] GnRH-agonist induced follicle > persistent first-wave ovulatory follicle; $P < .05$.

Discussion

Injection of a GnRH-agonist on d 9 after estrus induced ovulation of the first-wave dominant follicle (Experiment 1). Several reports indicate that a GnRH-agonist will ovulate a dominant follicle and cause atresia or luteinization of follicular structures of smaller size (Macmillan and Thatcher, 1991; Twagiramungu et al., 1994; Wolfenson et al., 1994). Continuous growth of the dominant follicle under low progestin environment preserves its ability to ovulate after exposure to a preovulatory surge of LH (Rajamahendran and Taylor, 1991). A first-wave dominant follicle grown in a low progesterone environment had more LH receptors (Cupp et al., 1993) and was more estrogenic (i.e., increased estradiol in follicular fluid; De la Sota et al., 1993) than a dominant follicle of similar age grown in a high progesterone environment. Injection of a GnRH-agonist (Buserelin) induces concurrent surges of FSH and LH (Chenault et al., 1990). Increased FSH induces recruitment of a follicular wave and programs emergence of a new ovulatory follicle (Adams et al.,1992; Badinga et al., 1992).

In the present study, synchronization of estrus was very good for both experimental groups with the incidence of estrus occurring within 3 d after $PGF_{2\alpha}$ injection and CIDR-B removal. The excellent synchrony may be due to several factors. Heifers entering the treatment sequence were synchronized with the first injection of $PGF_{2\alpha}$. This pretreatment eliminated prepubertal heifers and heifers between d 1 to 5 of the estrous cycle that would not respond to $PGF_{2\alpha}$. This preselection increased the chances of a good synchronization, because all heifers would have active follicles at d 16 when they were given $PGF_{2\alpha}$ and the CIDR-B device was removed. In T1, injection of the GnRH-agonist synchronized follicle recruitment as described in Experiment 1. Therefore, a homogeneous pool of ovulatory follicles was induced (Twagiramungu et al., 1994; Wolfenson et al., 1994). Such a follicular synchronization system before injection of $PGF_{2\alpha}$ and removal of the CIDR-B device should improve synchronization of estrus. Similarly, Adams (1994) reported that injection of estradiol valerate can induce recruitment of a new follicular wave in cattle when animals also are treated with a norgestomet implant. This could explain why an estradiol-valerate injection at the time of insertion of the norgestomet implant improves uniformity of estrus synchrony, compared with a synchronization protocol involving insertion of a norgestomet implant for 7 d and injection of $PGF_{2\alpha}$ at time of implant removal (Whittier et al., 1986). Absence of a corpus luteum at the time of removal of a progestin implant increases the number of animals in estrus during the next 3 d (Heersche et al., 1979). This is due partially to a reduced variability in the rate of decrease in plasma progesterone before estrus. A comparable relationship was possible in T2 of the present experiment. However, distribution of estruses differed between T1 and T2, and the extremely precise synchronization of estruses in T1 compared with T2 may be the result of synchronized follicular development compared with heterogeneity of follicular function among the persistent follicles of T2.

Fertility for the T2 group, with a programmed persistent first-wave dominant follicle, was reduced compared with the T1 group with a programed follicle that was induced by treatment with a GnRH-agonist. Savio et al. (1993b) previously reported reduced conception rates to AI at estruses induced by a persistent first-wave dominant follicle compared with AI at estruses induced by a 7-d-old first-wave dominant follicle. This reduced fertility was attributed to early embryonal mortality or failure of fertilization. Mihm et al. (1994) reported that oocytes collected from persistent dominant follicles underwent premature resumption of meiosis. Low fertility after ovulation of follicles with prolonged periods of dominance could be due to asynchronous nuclear and cytoplasmic maturation of the oocyte. Ahmad et al. (1995) reported retardation in embryonic development by d 6 after mating in cattle that ovulated a persistent first-wave dominant follicle. They suggested that increased plasma estradiol altered oocyte maturation or oviductal function. When a preovulatory follicle is maintained for 9 d in a low progestin environment, there is a normal pattern of synchrony between estrus behavior, LH surge, and ovulation (Rajamahendran and Taylor, 1991). Thus, failure to ovulate is not a causative factor of reduced fertility.

Injection of the GnRH-agonist on d 7 of the experimental sequence (T1) corrected the reduced conception and pregnancy rates observed in T2. As reported previously (Savio et al., 1993b), there is no apparent difference in interestrus intervals after AI of heifers that had not conceived as a result of the two treatments. This precluded the possibility that an alteration in corpus luteum lifespan (i.e., short cycles), might lead to embryonal mortality as described previously (Thatcher et al., 1994). An alteration in oviductal and uterine environments, induced by an extended proestrus period of high estradiol/low progesterone for heifers as treated in T2 (Savio et al., 1993a,b), could contribute to the reduced fertility. Based on ovarian changes characterized in Experiment 1, heifers in T1 of Experiment 2 should have ovulated the first-wave dominant follicle after injection of the GnRH-agonist and formed a corpus luteum. The induced corpus luteum should increase concentrations of plasma progesterone in T1 before the second injection of $PGF_{2\alpha}$ (Figure 3-3). Such an increase in plasma progesterone may modify the oviductal and uterine environments in a manner that is more favorable to development and growth of the embryo.

When animals are treated with a progesterone releasing device during the second part of the estrous cycle for estrus synchronization, the second- or third-wave dominant follicle may be maintained for an extended period of time after regression of the corpus luteum (Taylor et al., 1993). This may explain the reduced fertility when progestogens are used for an extended period of time (Odde, 1990) or when used during the second part of the estrous cycle for synchronization of estrus (Brink and Kiracofe, 1988). Injection of a GnRH-agonist, 7 d before the end of the progestogen treatment, could alleviate this problem by resynchronizing follicular growth, recruiting a new ovulatory follicle, and increasing potential for fertility, as demonstrated in the present experiment. Injection of a GnRH-agonist followed 6 or 7 d later by an injection of $PGF_{2\alpha}$ has been demonstrated to be a good system for estrus synchronization; 80% of the treated animals expressed estrus and conception rates were approximately 60% (Coleman et al., 1991; Twagimarungu et al., 1992b; Thatcher et al., 1993; Badinga et al., 1994). This is comparable to what is obtained with two injections of $PGF_{2\alpha}$ given 11 d apart (Coleman et al., 1991). However, the possibility of synchronizing estrus with two injections (GnRH-agonist and $PGF_{2\alpha}$) given 7 d apart makes this system simple to use under practical field conditions.

In summary, a GnRH-agonist (Buserelin, 8 µg) will ovulate a persistent first-wave dominant follicle maintained in a low progesterone environment and induce recruitment/selection of a new preovulatory dominant follicle. In a system designed to eliminate a persistent dominant follicle and induce a new preovulatory dominant follicle, synchronization of estrus was enhanced and fertility increased compared with responses induced with a 16-d-old persistent follicle.

Implications

Current methods of estrus synchronization in cattle essentially manage plasma concentration of progesterone with luteolytic agents and extended progestogens treatments. Incorporating treatments to synchronize follicle development (e.g., gonadotropin-releasing hormone agonist treatment) in concert with induction of corpus luteum regression should optimize precision of estrus and conception rates.

CHAPTER 4

DIFFERENTIAL RESPONSE OF THE LUTEAL PHASE AND FERTILITY IN CATTLE FOLLOWING OVULATION OF THE FIRST WAVE FOLLICLE WITH HUMAN CHORIONIC GONADOTROPIN VERSUS A GONADOTROPIN-RELEASING HORMONE AGONIST

Introduction

After intensive macroscopic and histological observations of ovaries, Rajakoski (1960) proposed the existence of two waves of follicular growth during the estrous cycle in cattle. These observations were confirmed with the ultrasonographic investigations of Savio et al. (1988), Fortune et al. (1988) and Ginther et al. (1989) to monitor ovarian follicular dynamics. It is now widely accepted that two to three waves of follicular growth occur in cattle during the estrous cycle. Each wave proceeds through stages of recruitment, selection and dominance (Ireland, 1987; Savio et al., 1988; Lucy et al., 1992) as described for primates (Goodman and Hodgen; 1983). The appearance and growth of the first follicular wave is very predictable in cattle (Savio et al. 1988; Badinga et al., 1992). Recruitment occurs after a rise in concentrations of plasma FSH (Adams et al., 1992; Sunderland et al., 1994) between d 0 and d 2 of the estrous cycle. Selection is characterized by the emergence of one estrogen active follicle (Ireland and

Roche, 1982, 1983a, b) on d 4 or 5 of the estrous cycle (Badinga et al., 1992; Sunderland et al., 1994). This follicle is dominant from d 5 to d 10 of the cycle when its size, as determined by ultrasonography, is at least 2 mm greater than any other follicle (Sirois and Fortune, 1990). During the period of dominance, growth of other follicles also is suppressed (Savio et al., 1988).

It was demonstrated previously that the first wave dominant follicle can ovulate after administration of GnRH (Rusbridge et al., 1992) or hCG (Price and Webb, 1989; Howard and Britt, 1990; Rajamahendran and Sianangama, 1992, Sianangama and Rajamahendran, 1992) given between d 4 and d 7 of the estrous cycle. These gonadotropin challenges induced formation of an accessory CL. The ability to induce an accessory CL during the early luteal phase of the estrous cycle in cattle could have some practical applications. Establishment and maintenance of pregnancy as well as embryo growth in cattle are related to the ability of the CL to secrete progesterone (Staples and Hansel, 1961; Estergreen and al., 1968). Several reports suggest that a deficiency in the steroidogenic secretion of the CL, leading to subluteal levels of progesterone, could be a factor contributing to embryo losses (Staples and Hansel, 1961, Thatcher et al., 1994). Cows that failed to conceive or lost an embryo had lower concentrations of progesterone in plasma and milk than cows that conceived (Henricks et al. 1971, Lamming et al., 1989). It also was suggested that the rate of increase in plasma progesterone during the early luteal phase could play a role in establishment of pregnancy (Shelton et al., 1990).

Indeed, several authors suggested that progesterone supplementation during the luteal phase of the estrous cycle could increase pregnancy rates in cattle (Johnson et al., 1958; Robinson et al., 1989, Macmillan et al., 1991; Sianangama and Rajamahendran, 1992) whereas others failed to show this effect (Van Cleeff et al., 1991; Walton et al., 1990), or obtained equivocal results with a tendency for improvement in pregnancy rates for the treated animals that was not significant statistically (Wiltbank et al., 1956). Geisert et al. (1988) showed that progesterone supplementation increased embryonic growth rate, and therefore its ability to secrete bovine interferon-τ.

Reduced fertility in cattle due to heat stress in summer is a well documented phenomenon (Thatcher and Collier, 1986). A diminution of the capacity of the corpus luteum to produce progesterone may be a contributing factor in the reduction of conception rates observed in dairy cows under heat stress conditions (Wolfenson et al. 1988; Wolfenson et al, 1993). Similarly, a reduction in the growth rate of the heat-stressed embryo, due to alterations of the uterine milieu in a subluteal environment, may impair its ability to synthesize the factors necessary for maintenance of the corpus luteum during early pregnancy (Putney et al., 1988a,b; Geisert et al., 1988). In both cases, progesterone supplementation after insemination in heat stressed cows, may correct the deleterious effects of impaired luteal function on pregnancy rates.

Inducing formation of an additional CL during the luteal phase of the estrous cycle with a gonadotropin challenge could be a strategy to increase concentrations of progesterone in plasma during the luteal phase. The estrogen synthesizing capacity of the first wave dominant follicle is maximal on d 5 of the estrous cycle (Badinga et al., 1992), and the number of LH receptors on theca and granulosa cells of the first wave dominant follicle increases from d 3 to d 7 of the estrous cycle (Ireland and Roche, 1983; Rolloson et al., 1994). On d 5 of the estrous cycle, migration of the embryo to the uterus is completed and should not be altered by induced variations of plasma concentrations of progesterone or estradiol at this time. These three factors make the first wave dominant follicle at d 5 of the estrous cycle a potentially optimal time for induction of an accessory CL.

Objectives of this series of experiments were to test whether a GnRH-agonist or hCG stimuli given on d 5 of the estrous cycle could cause ovulation of the first wave dominant follicle, and particularly to compare the effects of a GnRH-agonist vs hCG on subsequent concentrations of progesterone in plasma. In addition, effects of progesterone supplementation, via induction of an accessory corpus luteum with an injection of hCG, on the conception rates in heifers and lactating dairy cows during heat stress conditions were tested.

Materials and Methods

Experiment 1

The first experiment was designed to determine whether injection of a GnRH-agonist (Buserelin) given on d 5 of the estrous cycle would cause ovulation of the first wave dominant follicle, induce formation of an accessory CL, and if the plasma concentration of progesterone would be elevated during the luteal phase following injection of Buserelin. This experiment was conducted at the Dairy Research Unit of the University of Florida (Hague, FL). A group of 16 Holstein heifers between 12 and 14 mo of age were kept in a lot with minimal pasture and fed peanut hay and corn silage. Ovaries and reproductive tracts were palpated *per rectum* before initiation of treatment to confirm presence of a CL and to eliminate any heifers that presented gross morphological anomalies. Heifers (n=16) with a palpable CL were synchronized to estrus by insertion of a norgestomet ear implant (Syncro-mate B; implant containing 6 mg of norgestomet given without the injection of norgestomet/estradiol valerate; Sanofi Laboratories, Inc., Overland Park, KS) for 7 d. An injection of $PGF_{2\alpha}$ (Lutalyse®; 25 mg i.m.; The Upjohn Company, Kalamazoo, MI) was given 24 h before removal of the implant to induce regression of any endogenous CL. Animals were observed for estrus twice daily (0600 to 0700 h and 1800 to 1900 h) following removal of the implant. Tail heads were painted (Impervo, Benjamin Moore and Co., Montvalo, NJ) and chalked (All-weather

Paintstick, LA-CO industries inc., Chicago, IL) as an aid for estrus detection (Macmillan et al., 1988). When animals were observed in estrus, they were inseminated artificially and assigned randomly to two treatment groups. Heifers in T1 (treatment group; n=8) were injected on d 5 of the estrous cycle (d 0 = day of estrus) with a GnRH-agonist (Buserelin; 8 µg i.m.; Hoechst -Roussel Agri-Vet, Sommerville, NJ), whereas heifers of T2 (control group; n=7) were injected on d 5 of the estrous cycle with 2 ml of a sterile saline solution. Ten milliliters of blood were collected daily by jugular venipuncture into evacuated heparinized tubes (Vacutainer, Becton Dickinson Vacutainer systems, Rutherford, NJ) and immediately centrifuged (3000 *g* for 20 min). Plasma was separated and stored at -20°C until assayed for progesterone. Transrectal ultrasonographic examinations of the ovaries of each heifer were performed with an Equisonic LS 1000 linear-array ultrasound scanner equipped with a 7.5 MegaHertz Transducer (Tokyo Keiki Co., Ltd Tokyo, Japan) to confirm presence of a first wave dominant follicle (d 5), ovulation of the first wave dominant follicle (d 8), and formation of an accessory CL (d 15). Animals were checked for pregnancy by palpation *per rectum* 45 days after insemination.

Experiment 2

Ten heifers were selected and synchronized to estrus as in Experiment 1. Nine animals detected in estrus were assigned randomly, after artificial insemination, to one of two treatment groups, T1 or T2. In T1 (treatment group; n=5) heifers were injected on d 5 of the estrous cycle (d 0 = d of estrus) with an injection of hCG (1,000 IU i.v.; 2,000 IU, i.m.; Steris Laboratories, Phoenix, AZ). The hCG dose was split and 1,000 IU were given i.v. to simulate the rapid elevation in LH that occurs at the time of the ovulatory surge, and 2,000 IU were given i.m. to maintain levels of LH-like activity for an extended period of time. In T2 (control group; n=4) heifers were injected on d 5 of the estrous cycle with 2 ml of a sterile saline solution. Blood samples were collected and processed as in Experiment 1. Furthermore, blood samples for measurement of hCG were collected every 6 h starting before the injection of hCG to 42 h after treatment and one more sample was collected at 66 h. Two animals in T2 (treatment with saline on d 5) were sampled at 0, 12, 30 and 66 h after treatment with saline to establish baseline concentrations of hCG. Blood samples for hCG were centrifuged and plasma was separated and stored until further assay as described for progesterone. Ultrasonographic evaluation of the ovaries was performed on d 5, d 8 and d 15. Animals were checked for pregnancy by palpation *per rectum* 45 days after insemination.

Experiment 3

This experiment was designed to determine if the luteal phase plasma response of progesterone was greater following ovulation of the first wave dominant follicle on d 5 of the estrous cycle with an injection of hCG *versus* an injection of GnRH-agonist. Additionally, this experiment tested if a potentially reduced plasma progesterone response to an injection of GnRH-agonist given on d 5 of the estrous cycle could be corrected with an additional injection of hCG given soon after ovulation of the first wave dominant follicle.

Twenty five heifers were selected and synchronized as described in experiment 1. Twenty three animals were detected in estrus, artificially inseminated and assigned randomly to one of four treatment groups (T1, T2, T3 or T4). In T1 (n=6) heifers were injected on d 5 of the estrous cycle with 8 µg of Buserelin given i.m. In T2 (n=6) heifers were injected on d 5 of the estrous cycle with 1000 IU hCG given i.v. and 2000 IU hCG given i.m. as described for Experiment 2. In T3 (n=5) heifers were treated on d 5 of the estrous cycle with a GnRH-agonist as in T1 and received an injection of hCG (1000 IU i.v. and 2000 IU i.m.) 0 to 4 h after ovulation of the first wave dominant follicle. In T4 (n=6) heifers were treated with an injection of 2 ml of a sterile saline solution on d 5 of the estrous cycle. Collection and processing of blood samples as well as ultrasonographic evaluation of the ovarian structures (follicles and corpora lutea) was performed as for Experiments 1 and 2. Furthermore, ultrasonography was carried out every 4 h after injection of the GnRH-agonist for heifers in T3 to determine the approximate time of ovulation of the first wave dominant follicle. Animals were checked for pregnancy by palpation *per rectum* 45 d after insemination.

Experiment 4

This experiment was conducted at the heifer unit of a large commercial dairy in south Florida (Larson's Dairy, Okeechobee) to evaluate effects of an injection of hCG given on d 5 after insemination on the conception rates in heifers. Holstein heifers, 14 to 17 month old, weighing 350 ± 40 kg were used in this study. Animals were maintained on natural pasture and fed 3 kg of concentrate per animal per day. Heifers were synchronized with two injections of $PGF_{2\alpha}$ (Lutalyse, 25 mg i.m.) given 11 d apart. Detection of behavioral estrus was performed twice daily (0600 h and 1800 h) for 90 min. A modified tail-head chalk system was implemented for detection of estrus (Macmillan et al., 1988). All animals were bred with frozen/thawed semen from five bulls with known high fertility. Inseminations were performed by the same technician, once a day, after the morning detection of estrus (0730 h). Animals therefore were inseminated 0 to 12 h after estrus detection and 0 to 24 h after beginning of estrus behavior. This experiment was replicated twice. In the first replicate (April 1993), 131 heifers displayed signs of estrus during the 5 days after the second injection of $PGF_{2\alpha}$ and were allocated randomly to two treatment groups: T1 (n=66), heifers received an injection of hCG (1000 IU i.v., 2000 IU i.m.) 5 days after insemination, and T2 (n=65) heifers were treated with an injection of 2 ml of a sterile saline solution. In the second replicate, (June 1993) 112 heifers presented signs of estrus during the 5 d after the second injection of $PGF_{2\alpha}$ and were allocated randomly to T1 (n=56) or T2 (n=56) as described previously. Pregnancies were diagnosed by palpation *per rectum* 45 to 60 d after insemination.

Experiment 5

Experiment 5 was conducted in a commercial dairy of North Florida (Shenandoah Dairy, Live Oak) to evaluate effects of an injection of hCG given on d 5 after insemination in lactating dairy cows during the hot season (July 1994 to October 1994). Multiparous Holstein cows (n=201) in lactation for more than 55 d and which presented no anomalies of the reproductive tract, detectable by palpation *per rectum*, entered the experiment. Cows were fed a total mixed ration formulated according to NRC recommandations. Cows were milked three times daily and kept on pastures with free access to shaded barns equipped with sprinklers and cooling fans. Experimental animals were synchronized to estrus using an injection of GnRH-agonist (Buserelin, 8 µg i.m.) followed 7 d later by an injection of $PGF_{2\alpha}$ (Lutalyse®, 25 mg i.m.) as described (Thatcher et al., 1993). Detection of estrus behavior was performed by visual observation of the cows twice daily (0600 h to 0700 h and 1700 h to 1800 h) and the use of KAMAR heatmount detectors (KAMAR Marketing Group, Portland, ME). Cows that displayed signs of estrus during the 7 d following injection of $PGF_{2\alpha}$ were inseminated within 6 h after detection by one of three technicians using commercial semen from 45 different bulls. After breeding, cows were assigned randomly to one of two treatment groups (T1 or T2).

For T1 (n=99), animals received an injection of hCG (3000 IU i.m.) 5 or 6 d after insemination, and for T2 (n=102) animals did not receive any treatment on d 5 or 6 after insemination.

Determination of Plasma Progesterone and hCG

Concentrations of progesterone in plasma were determined by a single antibody RIA (Knickerbocker et al., 1986). Sensitivity of the assay was 156 pg/ml. Intra and inter-assay coefficients of variation were 6.8% and 12.5%. Pregnancies were diagnosed by palpation *per rectum* 45 to 60 days after insemination.

Concentrations of hCG in plasma were determined with the use of a commercial ELISA system (OPUS hCG, Behring Diagnostics Inc. Westwood, MA). Calibration was performed before assay, as described in the standard procedure, by assaying three commercial controls at three different levels throughout the dynamic range of the curve. The limit of detection of the assay is 1.3 mIU/ml. Intra-assay coefficient of variation was 7.4%. This assay was developed primarily for detection of hCG in human serum, and showed no measurable cross reactivity with human FSH, LH, and TSH at levels up to 500 mIU/ml. The baseline level of hCG observed in control cows or treated cows before treatment (n=13) was 15.42 ± 5.5 mIU/ml. This assay was not intended to be a precise measurement of hCG in plasma of heifers, but as a means of monitoring the presence of hCG activity in plasma over an extended period of time.

Statistical Analyses

Data were analyzed using the General Linear Model Procedures of the Statistical Analysis System (SAS, 1987). Discrete responses (formation of an accessory CL after treatment and conception rates) were compared by chi-square analysis and the categorical data procedures of the Statistical Analysis System.

Experiments 1, 2 and 3

Plasma concentrations of progesterone were analyzed using a mathematical model that examined the effects of treatment, pregnancy, cow(treatment - pregnancy), day and higher order interactions (Table 4-1). Because the effects of pregnancy and pregnancy x treatment on plasma concentrations of progesterone were not significant, a mathematical model that excluded the effects of pregnancy was used (Table 4-2). Error variances among treatments were heterogeneous for plasma concentrations of progesterone in Experiment 3. The data set was transformed ($\log_{10} y$) to recover homogeneity of variances. Plasma concentrations of progesterone are graphed untransformed and the respective standard errors of the means (SEMs) presented for each treatment. Differences between means in Experiment 3 were evaluated by preplanned orthogonal contrasts. Given the small number of animals in each experiment, the effect of treatment on conception rates was not analyzed.

Experiments 4 and 5

Conception rate was defined as the proportion of animals pregnant which were inseminated at a synchronized estrus. For Experiment 4, conception rates for the two replicates were analyzed with models including effects of treatment, bull, replicate, and the interactions treatment x bull, treatment x replicate, bull x replicate and treatment x bull x replicate. The effect of treatment was tested against the treatment x bull interaction; the effect of replicate was tested against the replicate x bull interaction; the replicate x treatment interaction was tested against the replicate x treatment x bull interaction. For Experiment 5, conception rates were analyzed with models including the effects of treatment, technician, bull, period (Before or after September,13 1994) higher order interactions. Since the effect of bull was not significant, a reduced model was analyzed that did not consider this effect.

Table 4-1. Least squares analysis of variance for the effects of treatment with a GnRH-agonist on day 5 of the estrous cycle on the plasma concentration of progesterone from d 6 to 16 of the estrous cycle

Source	d.f.	Mean Squares	Error term
Treatment	1	101.08	Cow (treatment*pregnancy)
Pregnancy	1	5.67	Cow (treatment*pregnancy)
Treatment*pregnancy	1	86.81	Cow (treatment*pregnancy)
Cow (treatment*pregnancy)	11	54.29	Residual
Day	10	72.25	Residual
Day*treatment	10	12.13 **	Residual
Day*pregnancy	10	4.72	Residual
Day*treatment*pregnancy	10	4.77	Residual
Residual	110	4.78	

** $P < .01$

Table 4-2. Reduced model for least squares analysis of variance for the effect of treatment on the plasma concentrations of progesterone from d 6 to 16 of the estrous cycle. (Experiments 1,2 and 3)

	Experiment 1		Experiment 2		Experiment 3		
Source	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.	Error term
Treatment	1	139.5	1	1127.14 **	3	268.78 *	Cow (Treatment)
Cow (treatment)	13	53.18	7	63.1	19	63	Residual
Day	10	78.33	10	108.58	10	224	Residual
Day * treatment	10	12.58 **	10	31.85 **	30	12.59 **	Residual
Residual	130	4.77	70	7.8	188	7.9	

* $P < .05$
** $P < .01$

Results

Experiment 1

All heifers treated with a GnRH-agonist injection on d 5 of the estrous cycle ovulated and formed an accessory CL (Table 4-3) as confirmed by ultrasonography (T1 [8/8] *versus* T2 [0/7], χ^2=15.15, $P < .01$). When ultrasonography was performed on d 15 it was not possible to distinguish between the original and induced CL based on diameter. There were no effects of pregnancy or pregnancy x day on the plasma concentrations of progesterone (Table 4-1) from d 6 to d 16 of the estrous cycle.

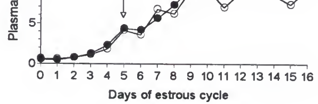


Figure 4-1. Least squares means (pooled SEM = .85 ng/ml) of plasma concentrations of progesterone for Holstein heifers treated with an injection of GnRH-agonist (Buserelin, 8 µg i.m.) or saline given on d 5 of the estrous cycle.

Table 4-3. Frequency of induced corpora lutea in each treatment group for the Experiments 1,2 and 3

Experiment	Treatment	Frequency of Induced Accessory Corpora Lutea	Conception Rate
Experiment 1	GnRH (n=8)	8 of 8[a]	5 of 8
	Control (n=7)	0 of 7[a]	4 of 7
Experiment 2	hCG (n=5)	5 of 5[b]	3 of 5
	Control (n=4)	0 of 4[b]	1 of 4
Experiment 3	GnRH (n=6)	5 of 6[c]	2 of 6
	hCG (n=6)	5 of 6[c]	1 of 6
	GnRH + hCG (n=5)	5 of 5[c]	2 of 5
	Control (n=6)	0 of 6[c]	5 of 6
Total (Experiments pooled)	GnRH (n=14)	13 of 14 (93%)	7 of 14 (50%)
	hCG (n=11)	10 of 11 (91%)	4 of 11 (36.4%)
	GnRH + hCG (n=5)	5 of 5 (100%)	2 of 5 (40%)
	Control (n=17)	0 of 17 (0%)	10 of 17 (58.8%)

[a] χ^2 =15.15; $P<.01$

[b] χ^2 =9.3; $P<.01$

[c] χ^2 =15.6; $P<.01$;

Orthogonal comparisons:

Effect of GnRH-agonist:

GnRH-agonist, GnRH-agonist /hCG *vs* hCG, control; $\chi^2 = 6.135$, $P < .01$

Effect of hCG:

GnRH-agonist /hCG, hCG *vs* control, GnRH-agonist; $\chi^2 = 6.135$, $P < .01$

Interaction of GnRH-agonist with hCG:

hCG, GnRH-agonist *vs* control, GnRH-agonist/hCG, $\chi^2 = 3.630$, $P < .05$

When the data set was analyzed from d 6 (24 h after treatment with GnRH-agonist or saline solution) to d 16 (Table 4-2), plasma concentrations of progesterone were altered by treatment (treatment x day interaction; $P < .006$). The injection of GnRH-agonist increased concentrations of plasma progesterone when compared to the control group (Figure 4-1); this effect was evident between d 11to d 16 (11.7 > 8.5 ± 1.0 ng/ml; $P < .05$).

Experiment 2

Injection of 3,000 IU of hCG on d 5 of the estrous cycle induced ovulation of the first wave dominant follicle and formation of an accessory CL (Table 4-3) in all heifers, whereas no heifers in the control group ovulated the first wave dominant follicle (T1 [5/5] *versus* T2 [0/4]; χ^2=9.3, *P* < .01). As in Experiment 1, the induced and original CL could not be differentiated when ultrasonography was performed on d 15. There was no effect of pregnancy or of the interaction of pregnancy with

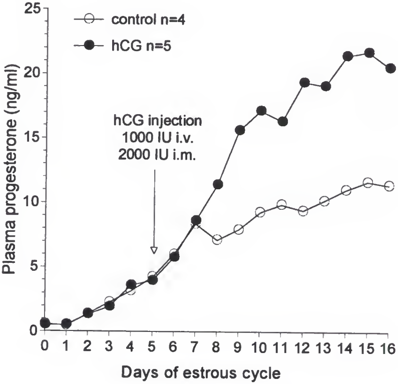


Figure 4-2. Least squares means (pooled SEM = 1.28 ng/ml) of plasma concentrations of progesterone for Holstein heifers treated with an injection of hCG (1000 IU, i.v.; 2000 IU, i.m.) or saline given on d 5 of the estrous cycle.

day on plasma concentrations of progesterone from d 6 to d 16 of the estrous cycle. Between d 6 (24 h after treatment with hCG or saline solution) to d 16 (Table 4-2), plasma concentrations of progesterone were altered by treatment (*P* < .004) and an interaction of treatment with day (*P* < .001). The injection of hCG increased the concentration of plasma progesterone compared to T2 (Figure 4-2); this effect was large between d 8 to d 16 (18.03 > 9.7 ± 1.2 ng/ml; *P* < .001). Plasma concentrations of hCG were elevated in T1 until 30 h after treatment (Figure 4-3).

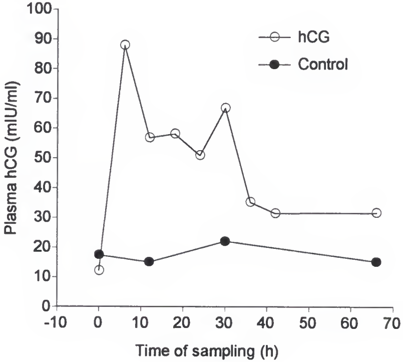


Figure 4-3. Least squares means of plasma concentrations of hCG (pooled SEM = 7.85 mIU/ml) as determined by ELISA, after treatment of 5 heifers with 3000 IU (1000 IU i.v.; 2000 IU i.m.) of hCG on d 5 of the estrous cycle. Control heifers (n=2) were treated with an injection of saline on d 5. Treated animals were sampled every 6 h from the time of injection until 42 h and one more sample was collected at 66 h. Samples in control animals were collected at 0, 12, 30, and 66 h.

Experiment 3

A majority of heifers treated with hCG (T2) or Buserelin (T1, T3) ovulated the first wave dominant follicle and formed an accessory CL (Table 4-3). The frequences of induced CL were: T1, 5 of 6; T2, 5 of 6; T3, 5 of 5; T4, 0 of 6 (χ^2=15.6; $P < .01$). As in the previous experiments, it was not possible to differentiate between the induced and original CL based on diameter determined by ultrasonography on d 15. There was no effect of pregnancy or of the interaction of pregnancy with day on the plasma concentrations of progesterone from d 6 to d 16 of the estrous cycle. Plasma concentrations of progesterone were altered by treatment ($P < .02$) and by the interaction of treatment with day ($P < .03$) when analyzed from d 6 (24 h after treatment with hCG, GnRH-agonist, GnRH-agonist and hCG or saline solution) to d 16 (Table 4-2). Plasma concentrations of progesterone (Figure 4-4) differed among treatments from d 8 to d 16 (T4 vs T1, T2, T3, $P < .006$; T1 vs T2, T3, $P < .02$; T2 vs T3, P > .41). Concentrations of progesterone between d 8 and d 16 were 10.1, 12.6, 13.9 and8.4 ± 1.1 ng /ml for T1, T2, T3 and T4. Residual error variances for T2 (7.7) and T3 (GnRH-agonist + hCG; 19.1) was larger than T2 (hCG; 7.7;$P < 0.01$) between d 6 to d 16. In T2 (hCG), responses to the treatment were more homogeneous than in T3 (GnRH-agonist + hCG); in T3, two of five heifers had plasma progesterone profiles comparable to heifers receiving GnRH-agonist alone and three heifers had profiles comparable to hCG (T2) administered alone (Figure 4-5).

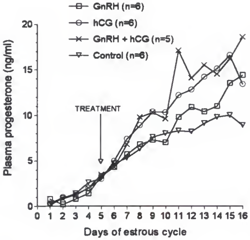


Figure 4-4. Least squares means (SEM ng/ml: hCG = .9, GnRH-agonist = .6, GnRH-agonist + hCG = 1.95, Control = .45) of the plasma concentrations of progesterone for Holstein heifers treated with injection of GnRH-agonist, hCG, GnRH-agonist followed 30 h later by hCG, or saline given on d five of the estrous cycle.

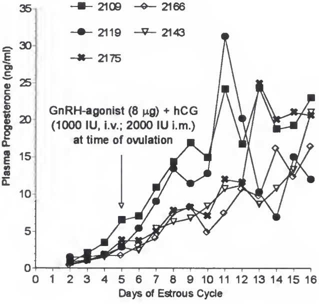


Figure 4-5. Effect of an injection of GnRH-agonist given on d five of the estrous cycle and followed by an injection hCG (1000 IU, i.v.; 2000 IU, i.m.) at the time of ovulation on individual profiles of plasma progesterone concentration for five Holstein heifers. Individual profiles of the 5 treated animals.

Experiment 4

Since no bull or replicate effects were detected, results from the two replicates were pooled together (Table 4-4).

Administration of 3,000 IU of hCG on d 5 after insemination in dairy heifers did not improve conception rates (hCG= 65%, control = 63%).

Table. 4-4. Conception rates of heifers treated with 3000 IU of hCG on d 5 after insemination (Experiment 4, replicates 1 and 2)

		Control [b]	hCG [b]
First replicate [a]	Number of heifers	65	66
	Conception rates at 45 days	66% (43/65)	70% (46/66)
Second replicate [a]	Number of heifers	56	56
	Conception rates at 45 days	59% (33/56)	59% (33/56)
Total	Number of heifers	121	122
	Conception rates at 45 days	63% (76/121)	65% (79/122)

[a] Conception rates did not differ between replicates, *P*<.19
[b] Conception rates did not differ between treatments, *P*<.98

Experiment 5

Conception rates were low in both groups due to effects of high temperatures and humidity during July to October. Conception rates were not improved (Table 4-5; *P*<.91) in T1 (hCG; 24.2%) when compared to T2 (Control; 23.5%). There was an effect of technician on conception rates (*P* < .05); one technician had a lower conception rate than the others, but the interaction of technician with treatment was not significant (*P* < .54). Conception rates were increased in the second part of the trial (from September 14th to October 25th) when temperature and humidity decreased at the end of the hot season (Table 4-5.). However there was no interaction of treatment with stage of the trial (i.e. before or after September 14th).

Table 4-5. Conception rates of lactating dairy cows treated with an injection of hCG (3000 IU i.m.) on d 5 or 6 after breeding (Experiment 5)

	Control	hCG
Number of cows	102	99
Conception rates at 45 to 60 days	23.5% [a] (24/102)	24.2% [a] (24/99)
Conception rates from July 19 to September 13, 1994	12.8% [b] (5/39)	9.3% [b] (4/43)
Conception rates from September 14 to October 25, 1994	30.1% [b] (19/63)	35.7% [b] (20/56)

[a] Conception rates do not differ with treatment, *P*<.91

[b] Conception rates are increased from September 14th to October 25th *P* < .05

Discussion

Injections of hCG or GnRH-agonist given on d 5 of the estrous cycle were successful in inducing ovulation of the first wave dominant follicle and forming an accessory CL. Fricke et al. (1993) reported that an injection of 1,500 IU of hCG given i.v. on d 6 of the estrous cycle induced ovulation of the first wave dominant follicle and formation of an accessory CL. Similarly, Price and Webb (1989) observed that 1,500 IU of hCG given i.v. elicited formation of an accessory luteal structure and at a higher frequency if given on d 4 to d 7 of the estrous cycle than during early (d 0 to d 3) or mid- (d 8 to d 13) luteal phases. Furthermore, Rusbridge et al. (1992) showed that administration of a synthetic GnRH (.5 mg) on d 6 of the estrous cycle could induce formation of an accessory CL in 75% of the animals. In the present study, 91% (10/11) of the animals treated with hCG and 93% (13/14) of the animals treated with Buserelin formed an accessory CL. Thus, the first wave dominant follicle at d 5 is very responsive for induction of an accessory CL. On d 15, similarly to what was observed by others (Price and Webb, 1989; Rajamahendran and Sianangama, 1992), it was not possible to distinguish between the original and the induced CL relative to size.

Injection of hCG or GnRH-agonist on d 5 of the estrous cycle increased plasma concentrations of progesterone. It was reported previously that an injection of hCG given on d 4 or d 7 (Breuel et al., 1989), d 2, d 3 and d 4 (Helmer and Britt, 1987), d 5 (Diaz et al., 1993) and d 6 (Fricke et al.,1993) would increase plasma concentrations of progesterone significantly compared to control. Similarly, Walton et al. (1990) reported that injection of hCG on d 5.5 of the estrous cycle increased concentrations of progesterone in milk between d 14 and d 20 after insemination. Of particular interest in the present experiment is the observation that heifers treated with hCG consistently had higher plasma concentrations of progesterone when compared to heifers treated with Buserelin. This may be due to differences in dynamics of LH-like exposure induced by these two pharmacological agents that results in induced CL with different steroidogenic capabilities. However, Webb et al. (1992) reported that GnRH induced corpora lutea developed into fully functional structures since their weight, progesterone and oxytocin contents, 7 days after induction, were identical to those of spontaneously formed corpora lutea of similar age. Perhaps hCG induces a greater luteinization of the preovulatory follicle and subsequent differentiation into an active CL, compared to Buserelin.

Administration of hCG or Buserelin also may have differential effects on the original CL that could account for differences in plasma progesterone. Veenhuizen et al. (1972) injected a single dose of hCG (1,000 IU, 2,000 IU or 4,000 IU) on d 3 after a synchronized estrus in heifers and observed an increase in size of the original CL removed on d 9, d 10 or d 11 of the estrous cycle. Furthermore, at the 4,000 IU dose of hCG, CL had a higher progesterone content (µg/g) and produced more progesterone in response to an hCG challenge in vitro. Responses to injection of hCG at d 3 supports the concept that LH-like exposure early in the development of the CL may stimulate CL development. Therefore, a d 5 injection of 3000 IU of hCG may induce modifications of the original CL as well as contributing to a greater development of the induced CL. The increase in plasma progesterone observed in the present experiment probably is due to combined effects on the original and induced CL.

In humans, the disappearance rates of LH and hCG in plasma show two phases that are different between LH and hCG. During the initial fast phase, LH has a half life of 21 min whereas hCG has a half life of 5 to 9 h. During the second phase of slow clearance, LH has a half life of approximately 4 h whereas hCG has a half life of 1 to 1.3 d. Therefore hCG is characterized by an extended plasma half-life when compared to LH (Yen et al., 1968). This difference is due to the greater content of sialic acid in hCG compared to LH that reduces hepatic uptake and therefore increases plasma half-life for hCG. In humans, i.m. injection allows a longer half life of hCG when compared to the i.v. route of administration (Rizkallah et al., 1969). Furthermore, Seguin et al. (1977) reported that injection of 10,000 IU of hCG in Holstein heifers doubled serum concentrations of LH for 10 h after treatment, as determined by a RIA for bovine-LH in which the antibody had a .1% crossreactivity to hCG. In Experiment 2, plasma concentrations of hCG were elevated markedly for 30 h after administration of hCG and had not returned to baseline concentrations at 66 h after treatment. Therefore a slow clearance of hCG in cattle is in accordance to what has been observed in humans. In comparison, administration of 10 µg of Buserelin during the luteal phase of the estrous cycle in heifers induced an elevation of LH in serum for approximately 5 h (Chenault et al., 1990). Perhaps the extended half life of hCG may induce a better maturation of the d 5 dominant follicle and formation of a CL with a greater steroidogenic capacity.

In addition, Mock and Niswender (1983) reported that times for internalization and degradation of radiolabeled hCG (22.8 ± 2.3 h) by ovine luteal cells are extended greatly when compared to ovine-LH (0.4 ± 0.2 h). This extended binding of hCG to the membrane of luteal cells could be responsible for the increased stimulatory effects of hCG on subsequent luteal phase concentrations of progesterone observed in the present experiment. In primates, induction of ovulation with GnRH or a GnRH-agonist promoted development of corpora lutea with suboptimal progesterone secreting capacities when compared to induction of ovulation with hCG (Zelinski-Wooten et al.,1991) in agreement with the present study.

Administration of hCG at the time of ovulation (Experiment 3) increased subsequent luteal phase concentrations of plasma progesterone in some of the GnRH-agonist treated heifers. Perhaps the additional LH-like stimulation via hCG promoted differentiation of follicular theca and granulosa cells into small and large luteal cells and a greater transformation of small to large luteal cells (Farin et al., 1988) that account for 80% of progesterone production in ovine CL (Niswender et al., 1985). The long half life of hCG in plasma combined with its persistence on the surface of the luteal cells when bound to receptors probably overcame any effect of endogenous LH pulses. Chronic administration of a potent LHRH-agonist every 6 h during 15 d increased the number of LH receptors in bull testis (Melson et al., 1986). Perhaps the long LH-like stimulation due to the persistence of hCG in plasma can upregulate the number of LH receptors in follicular or CL tissue. Such responses would increase the steroidogenicity of the hCG induced CL compared to the accessory CL induced by th GnRH-agonist.

The increased steroidogenic capacity of a CL obtained after ovulation of a first wave dominant follicle with an injection of hCG may have potential applications in reproductive management. Heifers can be inseminated artificially at a fixed time, eliminating the need for estrus detection when follicular growth and CL regression are synchronized by an injection of Buserelin (8 μg; i.m.) followed 7 d later by an injection of $PGF_{2\alpha}$ (Lutalyse® ,25 mg; i.m.) and ovulation induced by a second injection of Buserelin at either 24 or 48 h after $PGF_{2\alpha}$. Heifers were inseminated 15 h after the second injection of Buserelin (Chapter 7; Pursley et al., 1994). When this protocol is applied (Chapter 7) an increased frequency of shortened inter-estrus intervals ($\leq$ 16 d) was observed following timed-AI. Perhaps the second injection of a GnRH-agonist does not induce sufficient gonadotrophic stimulation of the pre-programmed ovulatory follicle. An ovulatory injection of hCG given 48 h after $PGF_{2\alpha}$ may lengthen CL lifespan and reduce the occurrence of short estrous cycles.

As mentioned previously, progesterone supplementation during the luteal phase of the estrous cycle may increase pregnancy rates in cattle (Johnson et al., 1958; Robinson et al., 1989; Macmillan et al., 1991; Sianangama and Rajamahendran, 1992). An injection of hCG on d 5 of the estrous cycle of the present studies induced ovulation of the first wave dominant follicle in a predictable and repeatable manner and led to the formation of an accessory CL capable of markedly enhancing plasma concentrations of progesterone. However, in Experiments 4 and 5 treatment of heifers or lactating dairy cows during heat stress conditions of summer failed to improve conception rates. These results contrast with those of Sianangama and Rajamahendran (1992) who reported an increase in conception rates when lactating dairy cows were treated with an injection of 1000 IU of hCG on d 7 or d 14 after insemination. Breuel et al. (1988) reported that an injection of 3000 IU of hCG on d 4 of the cycle preceding insemination and on d 4 after insemination increased conception rates in heifers. However, other studies did not show an effect of hCG (5000 IU) on d 3 after insemination (Helmer and Britt, 1986). Similarly, Breuel et al. (1990) showed that administration of a single i.m. administration of 3000 IU of hCG on day 4 after breeding did not improve conception rates in beef heifers. Wagner et al. (1973) reported a deleterious effect on conception rates when 2000 IU of hCG were administered to heifers 3 d after breeding at an estrus synchronized with 6-chloro Δ^6-17 acetoxyprogesterone. Plasma concentrations of progesterone may not be a limiting factor in establishment of pregnancy in fertile dairy heifers since conception rates were not further improved by progesterone supplementation via induction of an accessory CL in the present study

Several authors suggested that plasma progesterone levels may be lower in heat-stressed lactating dairy cows (Rosenberg et al., 1982; Wolfenson et al., 1988 and 1993). The data available on this subject is controversial since other reports show a slight (Thatcher and Collier, 1986; Wise and al., 1988) or a marked (Abilay and al., 1958) increase in plasma concentrations of progesterone. In the present experiment, administration of hCG (3000 IU) on d 5 or d 6 after breeding had no detectable effects on conception rates of lactating dairy cows exposed to elevated ambient temperatures of summer. Furthermore, administration of hCG did not improve conception rates when heat stress was maximal, (July 19 to September 13, 1994) or at the end of summer (September 14 to October 25, 1994) when temperature and humidity are decreased and conception rates start to improve (Thatcher and Collier, 1986). Since most of the damage to the conceptus in severly heat-stressed cattle occurs between the onset of estrus and d 7 of embryonic development (Putney et al., 1988a; Putney et al, 1989; Ealy et al., 1993), the rise in plasma concentrations of progesterone induced by the injection of hCG may have occurred at a time when survival of the embryo already was compromised. Reduced concentrations of progesterone in plasma may be a limiting factor in post-partum dairy cows that are not affected by heat stress. This could explain the positive effects of hCG on conception rates obtained with progesterone supplementation in lactating lactating dairy cows (Sianangama and Rajamahendran, 1992; Robinson et al., 1989) and would agree with observations of Lamming et al. (1989) that lactating dairy cattle experiencing embryonic losses, had significantly lower milk concentrations of progesterone from day 7 to 16 after insemination than animals that were inseminated and conceived or animals that were not bred.

Repeated use of hCG in cattle can induce formation of antibodies that can neutralize the hCG molecule and dramatically reduce its binding to receptors (Sundby and Torjesen, 1978). Thus, it would be appropriate to develop a pharmacological delivery system, involving a more prolonged delivery of GnRH to potentially lengthen the period of LH secretion from the pituitary. A normal period of LH secretion during the preovulatory surge of LH is 10 h (Chenault et al., 1975; Rahe et al., 1980) which is considerably longer than the 5 h surge elicited by an injection of GnRH or GnRH-agonist (Chenault et al., 1990). Insertion of a GnRH-agonist implant at the onset of estrus was associated with the development of of a more functional CL based on CL diameter and increases luteal phase progesterone concentrations in plasma during the subsequent cycle (Thatcher et al., 1993).

Implications

A GnRH-agonist or hCG are effective equally in ovulating a first wave dominant follicle at d 5 of the estrous cycle. Injection of hCG induced a greater stimulatory effect on luteal phase progesterone concentrations in plasma due to a greater stimulation of the original CL or an increased differentiation of the first wave dominant follicle into a more potent accessory CL. An injection of hCG on d 5 of the estrous cycle is an effective means to increase plasma concentrations of progesterone. However, such a treatment did not increase conception rates in lactating dairy cows during the heat stress conditions of summer or in fertile Holstein heifers. Additional research is warranted to stimulate the periovulatory period with enhanced LH availability to optimize subsequent CL development in programmed systems of ovulatory control associated with timed insemination or physiological periods (i.e. lactation) that may compromise subsequent CL development.

CHAPTER 5

A CELLULAR AND ENDOCRINE CHARACTERIZATION OF THE ORIGINAL AND INDUCED CORPUS LUTEUM AFTER ADMINISTRATION OF A GnRH-AGONIST OR hCG ON DAY 5 OF THE ESTROUS CYCLE

Introduction

The pattern of ovarian follicular growth in the bovine species has been characterized precisely with the use of ultrasonography (Savio et al., 1988; Fortune et al., 1988; Ginther et al., 1989). Two to three waves of follicular growth occur in cattle with the last wave producing the ovulatory follicle. During each wave, follicles undergo the processes of recruitment, selection and dominance as defined in the primate (Goodman and Hodgen, 1983; Lucy et al., 1992). Development of the first follicular wave is very predictable (Badinga et al., 1992; Lucy et al., 1992). The first wave dominant follicle can be identified as early as d 3 or d 4 of the estrous cycle or approximately 3 d after the recruitment phase triggered by the peri-estrous FSH surge (Adams et al., 1992; Sunderland et al., 1994). Growth of the first wave follicle continues until d 8 after estrus and maintains its size and dominance until at least d 10. On d 10 a new follicular wave is recruited as the first wave dominant follicle loses its dominance and a decrease in size has occurred by day 12 (Ginther et al., 1989; De la Sota et al., 1993). On d 5 of the estrous cycle, aromatase activity, estradiol content and gene expression of P450 SCC, 3β-HSD, P450 17α hydroxylase and P450 aromatase of the first wave dominant follicle are maximal and characterize a healthy steroidogenic active follicle (Badinga et al., 1992; De la Sota et al., 1995). Furthermore, dominant follicles possess the necessary enzymes to produce progesterone since they can synthesize this hormone when they undergo atresia (Ireland and Roche, 1983; Badinga et al., 1992; De la Sota et al., 1993). Other studies have shown that the number of LH receptors on the first wave dominant follicle is maximal during its growth phase (d 4 to d 8; Ireland and Roche, 1983; Rolloson et al., 1994). At the same time (d 5 to d 7 after estrus), the number of granulosa and thecal cells of the first wave dominant follicle reach maximum numbers (Ireland and Roche, 1983).

McNatty (1979), studying the human follicle, proposed three criteria for a functional follicle to be transformed into a corpus luteum (CL): 1) there must be sufficient granulosa cells in the follicle before ovulation (because after this event granulosa-lutein cell proliferation is absent); 2) the follicle must have granulosa cells with sufficient capacity or potential to produce progesterone; 3) granulosa as well as thecal cells must have a high receptor content for LH. Therefore, the first wave dominant follicle on d 5 after estrus should respond to preovulatory-like gonadotropin stimulus, ovulate and form a functional CL. Indeed administration of hCG (Price and Webb, 1989; Howard and Britt, 1990; Rajamahendran and Sianangama, 1992; Diaz et al., 1993; Fricke et al., 1993) between d 4 to d 7 of the estrous cycle induced ovulation of the first wave dominant follicle and formation of an induced CL. Similarly, injection of GnRH on d 6 (Webb, 1992) or a GnRH-agonist on d 5 (Schmitt et al., 1993) of the estrous cycle caused ovulation of the first wave dominant follicle with formation of an induced CL. Interestingly, when an induced CL was induced after administration of hCG or a GnRH-agonist on d 5 of the estrous cycle, plasma concentrations of progesterone for the hCG treated heifers were greatly increased compared to the GnRH-agonist treated heifers (Schmitt et al., 1993).

Objectives of the present experiments were: 1) to determine if injections of GnRH-agonist or hCG on d 5 of the estrous cycle had differential effects on the induced or original CL; 2) to assess in vitro progesterone production of the GnRH-agonist or hCG induced CL; 3) to perform an histological evaluation of the GnRH-agonist and hCG induced and original CL relative to number and percentage of different luteal cells among experimental corpora lutea.

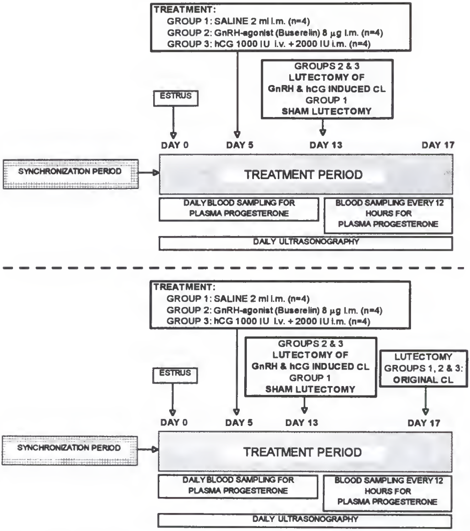
Materials and Methods

Animal Management

Both experiments were conducted at the Dairy Research Unit of the University of Florida (Hague). Non-lactating Holstein cows were kept in a lot with minimal pasture and fed peanut hay and corn silage. Ovaries and reproductive tracts were palpated *per rectum* before initiation of the treatment to confirm presence of a CL and eliminate any cow presenting gross morphological anomalies.

In the first experiment, cows (n=24) with a palpable CL were synchronized to estrus by insertion of a norgestomet ear implant (Syncro-Mate B; implant containing 6 mg of norgestomet; Sanofi Laboratories, Inc., Overland Park, KS), without the injection of norgestomet/estradiol valerate for 7 days with an injection of $PGF_{2\alpha}$ (Lutalyse®; 25 mg i.m.; The Upjohn Company, Kalamazoo, MI) given 24 h before removal of the implant. Animals were observed for estrus twice daily (0600 to 0700 h and 1800 to 1900 h) following removal of the implant. Tail heads were painted (Impervo, Benjamin Moore and Co., Montvalo, NJ) and chalked (All-weather Paintstick, LA-CO industries inc., Chicago, IL) as an aid for estrus detection (Macmillan et al., 1988). To ensure that ovulatory follicles would all be at the same stage of development and therefore not be an additional source of variation in the steroidogenic capacity of the original CL, cows were given a second injection of $PGF_{2\alpha}$ (25 mg i.m.) on d 6 after synchronized estrus to induce ovulation of a first wave dominant follicle at the same stage of follicular growth (Kastelic, 1990; Savio, 1990).

After detection of this second estrus, 12 cows were assigned randomly to three treatment groups (T1, T2, and T3; Figure 5-1). In T1 (n=4) cows were injected on d 5 (d 0 = day of estrus) of the estrous cycle with 2 ml of a sterile saline solution; in T2 (n=4) cows were injected on d 5 of the estrous cycle with a GnRH-agonist (Buserelin; 8 μg i.m.; Hoechst -Roussel Agri-Vet, Sommerville, NJ); in T3 (n=4) cows were treated on d 5 with an injection of hCG (1,000 IU i.v.; 2,000 IU, i.m.; Steris Laboratories, Phoenix, AZ). The hCG dose was divided with 1,000 IU given i.v. to simulate the quick elevation in LH that should occur at the time of the ovulatory surge, and 2,000 IU given i.m. to maintain the levels of LH-like activity for an extended period of time. On d 13, the induced CL was removed by flank laparotomy. Local and epidural anesthesia were performed using lidocaine (2% solution). A flank incision was made and the ovaries were visualized. After identification (Ireland et al., 1980), the induced CL was enucleated by exerting gentle pressure on the ovary. After bleeding from the ovary had stopped, the flank incision was sutured. The CL were transported immediately to the laboratory adjacent to the surgery room to be weighed and processed for culture of luteal slices and histological studies. Animals in the control group (T1) underwent a sham surgery, and a gentle pressure was exerted on the ovary bearing the original first wave dominant follicle, without damaging any follicular or luteal structures. Ten ml of blood were collected daily from d 0 to d 13 and every 12 h from d 13 to d 17 by jugular venipuncture, into evacuated heparinized tubes (Vacutainer, Becton Dickinson Vacutainer systems, Rutherford, NJ). Blood was centrifuged immediately (3000 *g* for 20 min). Plasma was separated and stored at -20°C until assayed for progesterone. Transrectal ultrasonographic examinations of the ovaries of each cow were performed daily, from d 0 to d 17 with an Equisonic LS 1000 linear-array ultrasound scanner equipped with a 7.5 megaHertz transducer (Tokyo Keiki Co., Ltd Tokyo, Japan) to confirm presence of a first wave dominant follicle, ovulation of the first wave dominant follicle and formation of an induced CL.


TREATMENT:
GROUP 1: SALINE 2 ml i.m. (n=4)
GROUP 2: GnRH-agonist (Buserelin) 8 µg i.m. (n=4)
GROUP 3: hCG 1000 IU i.v. + 2000 IU i.m. (n=4)
GROUPS 2 & 3
LUTECTOMY OF
GnRH & hCG INDUCED CL
GROUP 1
SHAM LUTECTOMY
ESTRUS
DAY 0
DAY 5
DAY 13
DAY 17
SYNCHRONIZATION PERIOD
TREATMENT PERIOD
DAILY BLOOD SAMPLING FOR PLASMA PROGESTERONE
BLOOD SAMPLING EVERY 12 HOURS FOR PLASMA PROGESTERONE
DAILY ULTRASONOGRAPHY
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GROUPS 2 & 3
LUTECTOMY OF
GnRH & hCG INDUCED CL
GROUP 1
SHAM LUTECTOMY
LUTECTOMY
GROUPS 1, 2 & 3:
ORIGINAL CL
ESTRUS
DAY 0
DAY 5
DAY 13
DAY 17
SYNCHRONIZATION PERIOD
TREATMENT PERIOD
DAILY BLOOD SAMPLING FOR PLASMA PROGESTERONE
BLOOD SAMPLING EVERY 12 HOURS FOR PLASMA PROGESTERONE
DAILY ULTRASONOGRAPHY

Figure 5-1. Experimental design for Experiment one (top panel) and Experiment two (bottom panel)

The second experiment was a replicate of the first experiment with 12 cows (Figure 5-1); however, the CL removed on day 13 were not studied. Instead, on d 17 the ovary bearing the original CL was removed by colpotomy (Drost et al., 1992). Following ovariectomy the CL was enucleated, transported to the laboratory and processed as for the first experiment.

Corpus Luteum Culture

After lutectomy, each CL was blotted dry, weighed and sliced into 300 to 400 mg slices with a Stadie-Riggs hand microtome (Thomas Scientific, Atlanta, GA) on a plane perpendicular to the surface of the CL. All slices from a CL were placed in a Petri dish (100 x 20 mm) containing 20 ml of Dubelcco's Modified Eagle's Medium without phenol red (DMEM). Slices were washed by gently shaking the dish, and then moved to another dish containing fresh medium. This process was repeated three times. Each slice was finally placed in an individual Petri Dish (60 x 15 mm) containing 6.0 ml of DMEM. Weight of CL tissue in each dish was adjusted to 250 mg. Eight slices were assigned randomly to one of four treatments in duplicate (one slice per dish): 1) control pre-incubation: 6 ml of absolute ethanol (4°C) was added to the culture dishes without incubation to stop progesterone synthesis (tissue and medium were immediately stored at -20°C); 2) control post-incubation: incubation with no treatment; 3) incubated with bovine-LH (b-LH; 20 ng/ml). The b-LH (USDA B-5, Beltsville, MD) was reconstituted in DMEM (100 ng/ml) and added to the culture to obtain a total volume of 6 ml; 4) incubated with b-LH (40 ng/ml). Petri dishes of the control post-incubation and the two b-LH treatments were incubated for 2 h (37°C; 5% CO_2). At the end of the incubation period, absolute ethanol (6 ml, 4°C) was added to each Petri dish to terminate progesterone synthesis, and samples (culture medium + luteal slice + ethanol) were stored at -20°C until further assay of progesterone content.

Extraction of progesterone from the CL slices was performed according to the procedure described by Martin et al. (1990) and Slayden and Stormshak (1990). Each sample had [3H]-progesterone (45,000 dpm) added before homogenization to assess procedural losses. Tissue plus medium in absolute ethanol were homogenized in a Duall 24 ground glass homogenizer. Homogenized samples were filtered through Whatman no. 1 filter paper. Homogenizer and filter were washed with an additional 5 ml absolute ethanol. Filtered samples were evaporated to dryness under a gentle air stream in a 45°C water bath. Samples were reconstituted in 1 ml PBS, vortexed, allowed to equilibrate for 30 min and extracted with 10 ml hexane/benzene (2:1). Samples were stored at -20°C overnight; the organic solvent was decanted and dried in a water bath at 45°C. The solvent residue was redissolved in 10 ml absolute ethanol and 500 µl quantified for radioactivity, after addition of 4 ml Scintiverse™ II (Fisher Scientific, Pittsburgh, PA) to estimate procedural losses. Average recovery was 87.3 ± 5.8 %. From the final redissolved sample (10 ml), 100 µl was diluted (1/400) in PBS and 50 µl of the final dilution were assayed for progesterone.

Histology

Light microscopy. Fresh CL slices (1 mm thick) were dissected in 5 x 5 mm pieces, fixed overnight in neutral buffered formalin (Sheenan and Hrapchack, 1980) and embedded in paraffin. Four sections (5 µm thickness) were cut from the paraffin blocks such that each section was from a different region of the block. The sections were placed on glass slides and stained with hematoxylin and eosin (Sheenan and Hrapchack, 1980) for morphological evaluation. Three areas of each section were photographed with a light photomicroscope and the negatives printed to a final magnification of 300x. Each photograph (n=12/animal) represented an area of 17,250 μm^2. Various luteal cell parameters were evaluated from these photographs. First, size of 100 large and small luteal cells and the number of cells in each size category was determined. Small luteal cells were considered to be those < 20 μm in diameter, while large luteal cells were considered those > 20 μm in diameter. Second, the number of small and large luteal cells per surface area of each section was determined. Measurements were conducted with a Zeiss Videoplan (Georgia Instruments, Inc, Atlanta, GA).

Electron Microscopy. Luteal tissues were processed for electron microscopic evaluation as described previously (Fields et al., 1992). Briefly, the tissue was placed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight at 4°C, rinsed with cacodylate buffer and post-fixed with 2% osmium tetroxide (2 h). Following rinsing with cacodylate buffer, the tissues were dehydrated with successive washings of ascending concentrations of ethanol (50% to 100%), ethanol:acetone (1:1), acetone (100%), acetone:Spurr's plastic (1:1), Spurr's plastic (100%), and embedded in Spurr's plastic. Ultrathin sections (.1 μm thickness) were cut, placed on copper grids, and stained with lead citrate and uranyl acetate. Luteal cells from the stained sections were photographed with Phillips 301 electron microscope (Phillips Electronic Instruments, Mahwah, NJ) in order to evaluate the morphology of the luteal cells. Small and large luteal cells were categorized within three stages of development. Percentages of cells within each stage were determined by electron microscopic evaluation of 150 large and small luteal cells from corpora lutea of each treatment; i.e. 50 large and small luteal cells each from CL of each of three cows per treatment.

Progesterone Radioimmunoassay

Radioimmunoassay for progesterone in plasma followed the protocol described by Knickerbocker et al. (1986). The assay level of detection was 0.2 ng/ml. Intra- and inter-assay coefficient of variation were 8.5 % and 6.5 %. For the progesterone content of luteal slices, 200 µl of the diluted samples were assayed. High and low reference samples were generated by adding respectively 480 µg and 40 µg of progesterone (Steraloids Inc., Wilton, NH) to 100 ml absolute ethanol to obtain a final concentration after dilution in PBS (1/400) of 12 ng/ml (High) and 1 ng/ml (Low) of progesterone. Samples were assayed (50 µl) without the extraction procedure described by Knickerbocker et al. (1986). Intra- and inter-assay coefficient of variation were 7.29% and 3.9%.

Statistical Analyses

Data were analyzed using the General Linear Model Procedure of the Statistical Analysis System (SAS, 1987). Plasma concentrations of progesterone were analyzed using a model that examined the effects of treatment, experiment, cow(treatment experiment), day of the experiment and higher order interactions; since cow was considered as a random effect, the effect of treatment and experiment were tested against cow(treatment experiment). Variances were not homogeneous for the different treatments ($P < .001$) and a $\log_{10}$ transformation was applied to the data in order to establish homogeneity of variances. No effect of experiment or experiment x treatment interaction was detected. In order to estimate the contrast to compare the means of the different treatments, a reduced mathematical model that excluded the effect of experiment was used. For ease of presentation, data are plotted untransformed and the respective SEMs presented for each treatment. Progesterone production by CL slices was analyzed with a model that examined the effects of treatment, cow(treatment), dose of LH and the interaction of treatment with dose. The size of small and large luteal cells as evaluated by light microscopy, were analyzed for the effect of treatment and cow(treatment). Number of luteal cells per surface area was analyzed with a mathematical model that examined effects of treatment, cow(treatment), class (i.e. small or large luteal cells), photographic replicate(cow treatment) and higher order interactions. The percentage of small and large luteal cells within each stage of development as evaluated by electromicroscopy, was analyzed with a mathematical model that examined the effect of treatment, cow(treatment), stage of development, class (i.e. small or large luteal cells) and higher order interactions.

Results

Corpus Luteum Weight

In both experiments, all heifers treated with a GnRH-agonist or hCG injection on d 5 of the estrous cycle ovulated and formed an induced CL as confirmed by ultrasonography. Administration of hCG induced heavier induced CL when compared to the induced CL formed after injection of GnRH-agonist (*P* < .04; Table 5-1). Weights of original CL removed on d 17 after treatments did not differ between treatments (Table 5-1).

Table 5-1. Weight of CL removed on d 13 (induced CL) and d 17 (original CL) after estrus.

Type of CL	Treatment	CL wt (g)
Induced CL removed on d 13	GnRH-agonist (T2)	3.67^a
	hCG (T3)	5.34^b
	SEM	.45
Original CL removed on d 17	Control (T1)	5.63
	GnRH-agonist (T2)	7.86
	hCG (T3)	8.22
	SEM	1.24

$^{a, b}$ Means with different superscripts differ; *P* < .05

Plasma Concentration of Progesterone

When the data set was examined from d 0 to d 17, d 0 to d 5, d 6 to d 13 and d 14 to d 17, there was no effect of experiment or interaction of experiment with treatment on plasma concentrations of progesterone. From d 0 to d 5, progesterone did not differ between the three treatments as expected (Figure 5-2). From d 6 to d 13, the linear rate increase of plasma progesterone (ng $ml^{-1}d^{-1}$) differed between treatments (Control [1.1]< GnRH-agonist [1.54] < hCG [2.63]; *P*

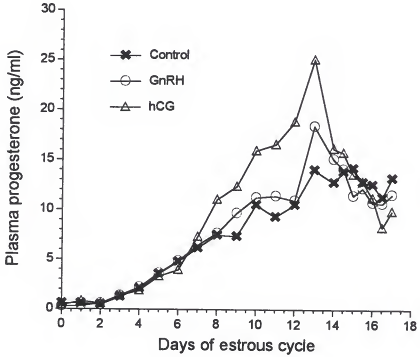


Figure 5-2. Least square means estimate (pooled SEM = .59 ng/ml) of the pooled plasma concentrations of progesterone for Experiments 1 and 2.

< .01). On d 13, before removal of the induced CL, average plasma progesterone differed between the three treatment groups (T1 [13.8±.92] < T2 [18.3±.92] < T3[24.7±1.1]; p<.01). From d 14 (24 h after removal of the induced CL) to d 17 there was no effect of treatment or the interaction of treatment with day on plasma progesterone.

Progesterone Production in Vitro

Induced CL day 13. There was no treatment difference in concentrations of progesterone of luteal slice before incubation (Figure 5-3). However, the quantity of progesterone extracted from the slices of induced CL and secreted into the culture medium (µg per g of CL tissue) after 2 h of incubation was influenced by treatment (GnRH-agonist [110.7 ± 15.8] vs hCG [172.0 ± 15.8]; *P* < .04) and dose of LH (ng/ml) in culture medium (0 [111.5 ± 12.3] vs 20 and 40 [156.43 ± 12.3];*P* < .02). There was no interaction of treatment by dose of LH, the concentration of progesterone in the hCG induced CL being always higher than for the GnRH-agonist induced CL for all doses of LH in culture medium. When progesterone extracted from luteal slices before incubation was adjusted for total weight of CL (Figure 5-3), treatment with hCG increased the progesterone content (µg/CL) when compared to GnRH-agonist (T3 [538.5 ± 67.2] > T2 [267.2 ± 65.8]; *P* < .02). Similarly, after 2 h of incubation the CL progesterone content increased with LH dose in culture medium (0 [533.3 ± 68.1] < 20 and 40[741.4 ± 68.1] ng/ml; *P* < .06) and was greater in hCG treated cows (T3 [937.3 ± 119.3] < T2 [406.8 ± 119.3]; *P* < .02). However, there was no interaction of treatment with dose on CL content.

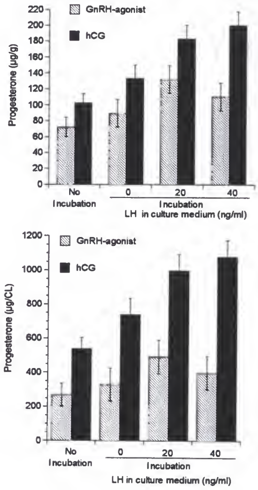


Figure 5-3. Top panel: Quantity of progesterone extracted from the luteal slices and culture medium with and without a 2 h incubation in culture medium per g of induced CL tissue at d 13 of the estrous cycle. Luteal tissue was incubated for 2 h in culture medium containing different concentrations of bovine-LH. Concentrations of progesterone differed with treatment (P < .04) and dose of LH in culture medium after a 2 h incubation (*P* < .02) but no differences were detected before incubation. Bottom panel: quantity of progesterone extracted was adjusted for luteal weight (bottom panel). Total progesterone content differed before incubation (*P* < .02). Similarly progesterone content of the CL after 2 h incubation differed with treatment (*P* < .02) and was increased with the LH doses in culture medium (*P* < .06).

Original CL day 17. There was no effect of treatment on progesterone concentration of CL slices (µg/g) before or after incubation (Figure 5-4). The

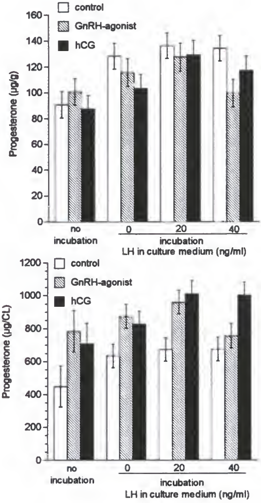


Figure 5-4. Top panel: quantity of progesterone extracted from the luteal slices and culture medium per g of original CL tissue at d 17 of the estrous cycle. Luteal tissue was incubated for 2 h in culture medium containing different concentrations of LH. Progesterone did not differ with treatment or dose of LH in culture medium.

Bottom panel: quantity of progesterone extracted was adjusted for luteal weight. Total progesterone content differed before incubation ($P < .08$). No differences were detected after incubation among treatment groups.

original CL removed on d 17 did not respond to in vitro incubation with LH for a 2 h period. Progesterone content of CL tissue (µg / CL) prior to incubation was increased by treatment with hCG or GnRH-agonist (T1 [449.4 ± 125.1] < T2, T3 [746.4 ± 125.1]; *P* < .08). However, no effect of treatment or dose of LH in the culture medium was detected when the quantity of progesterone extracted from the luteal slices and secreted in the medium was expressed per total weight of CL (Figure 5-4).

Light Microscopy

Induced CL day 13. There were no differences in the number of small or large luteal cells per 17250 µm^2 of CL induced after treatment with GnRH-agonist or hCG (Table 5-2, Figure 5-5). However, the average size of small luteal cells was increased for CL induced with hCG (13.5 ± .37 µm) as compared to induced by GnRH-agonist (10.3 ± .34 µm; *P* < .001). There was no difference in the average size of large luteal cells between the two treatments.

Original CL day 17. No differences were detected in the number of small or large luteal cells from original CL exposed to either saline, GnRH-agonist or hCG on d 5 (Table 5-2, Figure 5-6). However, treatment with GnRH-agonist and hCG increased average size for small luteal cells (Control [11.6 ± .2] < GnRH-agonist [12.3 ±.2] < hCG [13.1 ± .2]; *P* < .02) and large luteal cells (Control [28.1 ± 1.2] < GnRH-agonist [31.4 ± 1.2], hCG [30.9 ± 1.2]; *P* < .06).

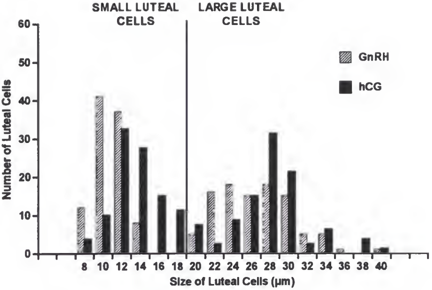


Figure 5-5 . Number of luteal cells by size class for the induced corpus luteum removed on day 13 of the estrous cycle: Counts of 100 small and 100 large luteal cells organized by 2 µm size class. Observation of 175250 µm^2 area (one slide by cow).

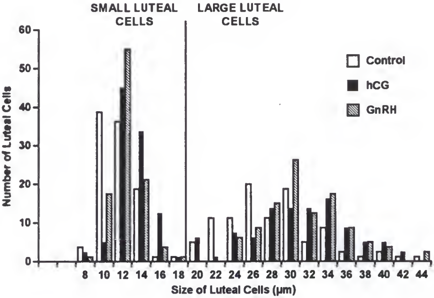


Figure 5-6 . Number of luteal cells by size class for the original corpus luteum removed on day 17 of the estrous cycle: Counts of 100 small and 100 large luteal cells organized by 2 µm size class. Observation of 175250 µm^2 area (one slide by cow).

Table 5-2. Average number of small (8 to 20 µm) and large (> 20 µm) luteal cells over a 17250 µm^2, and average size (µm) of the small and large luteal cells for the induced CL removed on d 13 and the original CL removed on d 17.

	Number of luteal cells over a 17250 µm^2 area (12 samples / CL)		Size of small and large luteal cells	
	Average number of small luteal cells	Average number of large luteal cells	Average size of small luteal cells (µm)	Average size of large luteal cells (µm)
Induced CL removed on d 13 after estrus				
GnRH-agonist	126.4	17.3	10.3^{b}	26.6
hCG	125.4	13.8	13.5^{a}	28.1
SEM	3.3	3.3	.3	1.1
Original CL removed on d 17 after estrus				
Control	93.2	22.8	11.6^{c}	28.1^{f}
GnRH-agonist	81.3	20.8	12.3^{d}	31.4^{g}
hCG	92.2	17.6	13.1^{e}	30.9^{g}
SEM	4.7	4.7	.2	1.2

$^{a,\,b}$ Means with different superscript differ ($P < .001$)

^{c,d,e} Means with different superscript differ ($P < .02$)

^{f,g} Means with different superscript differ ($P < .06$)

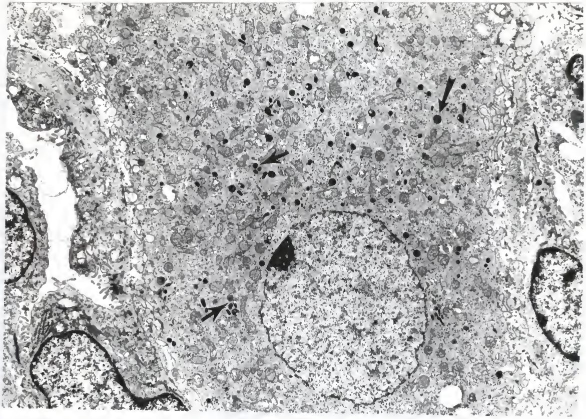
Electron Microscopy

Two types of luteal cells, large (> 20 µm diameter) and small (8 to 20 µm diameter) were identified. These cells appeared to go through three stages of development leading up to a fourth stage or regression. Although there are no

Figure 5-7. Stage 1 luteal cells.

A) Upper panel: large luteal cell with characteristic oval nucleus, intact mitochondria, numerous dense secretory granules (arrows) and an undiscernible smooth endoplasmic reticulum. One cell fills the field at 5,000 magnification [treatment = hCG induced; #50426]

B) Lower panel: two small luteal cells with similar characteristics described for the large cell except for the presence of junctional complexes (arrows) between the small cells. x 5,000 [treatment = GnRH induced; #50345].



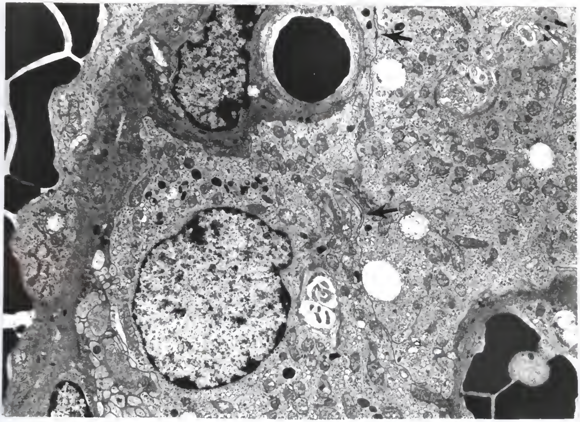


Figure 5-8. Stage 1 small luteal cells connected by desmosomes (large arrow). x 5,000. Insert is a high magnification of the desmosomes with their dense plaques. x 38,000. Note lipid droplets (L) and dense granules (small arrows) [treatment = hCG original; #50805]

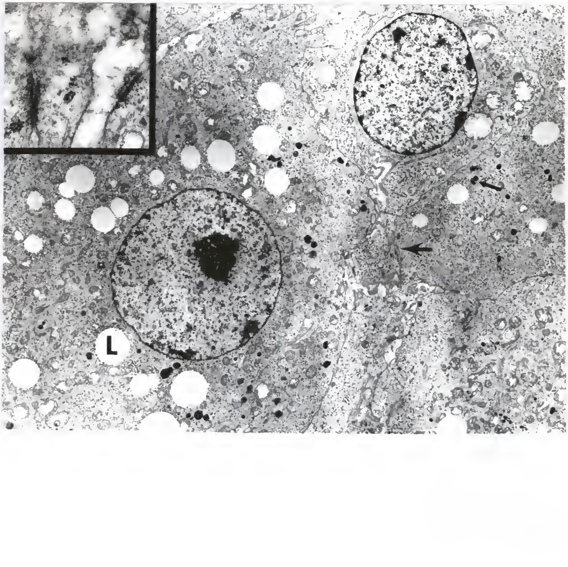
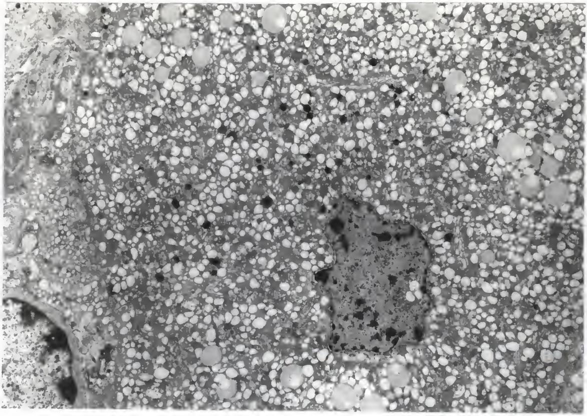

L

Figure 5-9. Stage 2 luteal cells.

A) Upper panel: large luteal cell with pleomorphic shaped nucleus, intact mitochondria, dense secretory granules, and distended smooth endoplasmic reticulum (arrows). x 5,000 [treatment = GnRH original; #50292]

B) Bottom panel: two small luteal cells with pleomorphic shaped nucleus, intact mitochondria and distended smooth endoplasmic reticulum (arrows). x 6,800 [treatment = hCG induced; #50325]



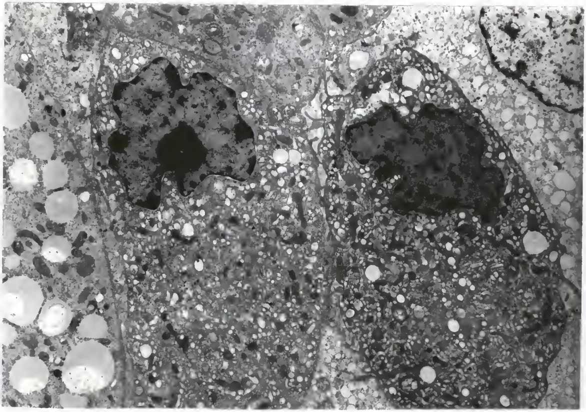


Figure 5-10. Stage 1 and Stage 2 luteal cells.

A) Upper panel: Stage 1 (right side) and Stage 2 (left side) large luteal cells adjacent to one another. x 3,800 [treatment = GnRH original; #50339]

B) Lower left panel: Stage 1 small luteal cell (left center) surrounded by five Stage 2 small luteal cells. x 3,800 [treatment = GnRH original; #50294]

C) Lower right: Mitochondria of a stage 2 large luteal cells, some of which contain dense osmiophilic material. x 30,000 [treatment =hCG original; #50848]

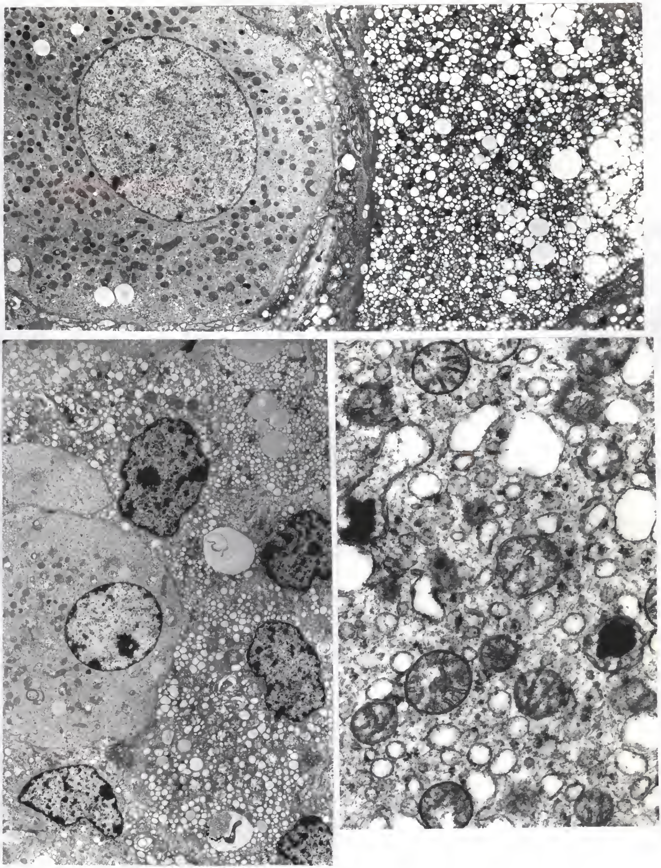
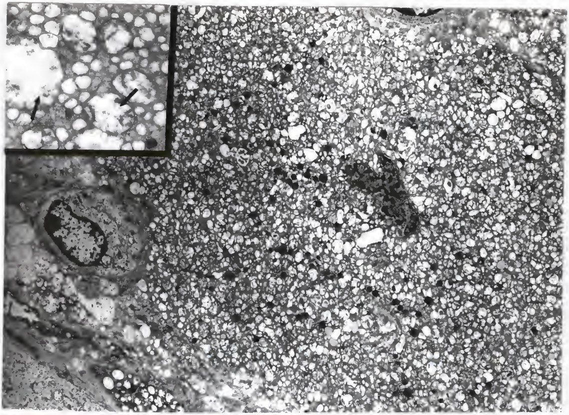
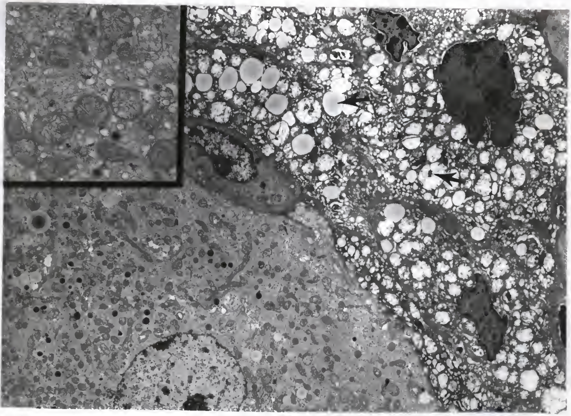


Figure 5-11. Stage 3 luteal cells.

A) Upper panel: Large luteal cell with a greatly distorted nucleus, swollen smooth endoplasmic reticulum and swollen mitochondria with disrupted cristae. Note secretory granules (arrow). x 3,800. Insert - high magnification of swollen smooth endoplasmic reticulum (small arrows) and swollen mitochondria (large arrows) with disrupted cristae. x 15,000 [treatment = control; #insert 50855; #50231]

B) Lower panel: Four Stage 3 small luteal cells (left side) with swollen endoplasmic reticulum and swollen mitochondria with washed out cristae (large arrows). A stage 1 large luteal cell (lower left) adjacent to the small cells. Insert - high magnification of the intact mitochondria of the luteal cell. x 15,000 [treatment = control; #insert 50855; #50322]





markers to demonstrate that there are only one type of small and large luteal cell that progress through these three stages, the changes in percent of cells within each stage correlates with age of corpus luteum and treatments.

Stage 1 luteal cells are the most prominent cells in the induced CL which is an 8-day old CL. Large luteal cells (Figure 5-7A) contain numerous mitochondria with intact cristae, a smooth endoplasmic reticulum which is indistinct or slightly distended, and an oval nucleus. A distinct cluster of secretory granules was observed in 95 % of these cells. Small luteal cells (Figure 5-7B) look like Stage 1 large cells except for their smaller size, and less mitochondria and secretory granules. There are junctional complexes between adjacent small cells (Figure 5-8) which are not observed between large cells.

Stage 2 luteal cells were most prominent in the original CL of hCG and GnRH-agonist treated cows compared to control CL on d 17 (Table 5-3). The large and small luteal cells of stage 2 have a more dilated smooth endoplasmic reticulum than do those of Stage 1 and the nucleus is more pleomorphic in shape (Figure 5-9). Stage 1 and 2 cells can be found adjacent to each other (Figure 5-10A and 5-10B), indicating that the morphology reflects inherent properties of the cell in response to processing procedures. The mitochondria of the large luteal cells designated as Stage 2 have begun to accumulate electron dense material (Figure 5-10C), furthermore, 90% of the Stage 2 large luteal cells still contain a cluster of secretory granules.

Stage 3 luteal cells were the most abundant cell type in the CL of the d 17 untreated cow (Table 5-3) and appear to be in the final stages of cell involution prior to Stage 4 (regression). Stage 3 cells have a very dilated smooth endoplasmic reticulum, a nucleus with a greatly distorted shape, and dilated mitochondria with undiscernible cristae (Figure 5-11A). A cluster of secretory granules was observed in less than 50 % of the large luteal cells in these cows. The presence of a Stage 1 luteal cell adjacent to Stage 3 cells again indicates that the morphology is due to inherent differential properties of the cells in response to fixation and embedding (Figure 5-11B).

The induced CL (8-day old CL) induced with hCG and GnRH-agonist contained more than 80% Stage 1 luteal cells (Table 5-3). The remaining cells were characteristic of Stage 2. There was a treatment x stage interaction, the hCG induced CL presented a slightly greater proportion of Stage 1 small and large luteal cells than the GnRH-agonist induced CL ($P < .1$). The original CL from hCG and GnRH-agonist treated cows (d 17 of the estrous cycle) contained small and large luteal cells that were less than 20% Stage 1, 60 to 69% Stage 2 and 20% to 29% Stage 3 luteal cells. In contrast CL from untreated cows on day 17 of the estrous cycle contained small and large luteal cells that were less than 10% Stage 1, 35% Stage 2, and approximately 60% Stage 3 (treatment x stage: $P < .001$; Table 5-3).

Table 5-3. Effects of treatment on the percent of luteal cells at each stage.

Treatment/CL	% of Luteal Cells at Each Stage[a]					
	Stage 1		Stage 2		Stage 3	
	LLC	SLC	LLC	SLC	LLC	SLC
hCG / induced[b]	87%	90%	13%	10%		
GnRH-agonist / induced[b]	84%	85%	16%	15%		
hCG / original[c]	11%	12%	69%	67%	20%	20%
GnRH-agonist / original[c]	9%	10%	62%	60%	29%	29%
Untreated / original[c]	7%	8%	35%	33%	58%	59%

[a] Percentages determined by electron microscopy evaluation of 150 large and small luteal cells from corpora lutea of each treatment; i.e. 50 large and small luteal cells each from CL of each of three cows/treatment.

[b] Treatment x stage: $P < .1$

[c] Treatment x stage: $P < .001$

Discussion

Fricke et al. (1993) hypothesized that most of the progesterone increase observed from d 8 to d 13 after treatment with hCG on d 6 of the estrous cycle was due to an effect of hCG on the original CL. Results of the present experiment do not agree with this hypothesis. The elevation in plasma concentrations of progesterone after induction of an induced CL, with administration of a GnRH-agonist or hCG on d 5 of the estrous cycle, appears to be due to the progesterone produced by the induced CL. This is implied, since, plasma progesterone did not differ among the three treatment groups after removal of the induced CL. Effect of hCG or GnRH-agonist administration on the original CL should not be excluded (Veenhuizen et al., 1972; Martin et al., 1990), but the consequences of these effects on plasma progesterone could not be detected between d 14 to d 17 with the present experimental design.

There is little information on plasma concentrations of progesterone after induction of an induced CL with an injection of a GnRH-agonist in cycling cows. Twagiramungu et al. (1995) did not detect any increase in plasma progesterone during the 6 d after induction of an induced CL with administration of a GnRH-agonist on d 7 after estrus. Martin et al. (1990) administered 100 µg of natural GnRH on d 2 and d 8 of the estrous cycle and did not detect any increase in plasma progesterone in the treated animals, but failed to mention whether induction of induced CL occurred. In the present experiment, rate of increase of plasma progesterone from d 6 to d 13 in GnRH-agonist treated animals was greater than for control. Therefore, the suppressive action of GnRH administered earlier during metestrus on CL function (Ford and Stormshak, 1978; Lucy and Stevenson, 1986; Rodger and Stormshak, 1986) was not observed in this study. The increased steroidogenicity of the hCG-induced CL may be due to different reasons. Administration of hCG induces an increase in LH-like activity (Seguin et al., 1977; Schmitt et al., 1995) for a longer period of time (30 h) when compared to the length of the LH-surge (5 h) elicited by an injection of a GnRH-agonist (Chenault et al., 1990). Furthermore, the rate of internalization of hCG bound to the LH receptor on ovine luteal cells ($t½ = 22.8$ h) is much slower than that of ovine-LH ($t½ = 0.4$ h). Since internalization of the LH receptor bound to its ligand is the mechanism used by the luteal cell to terminate a response to the gonadotropin hormone (Niswender et al., 1985), the slower rate of internalization of the hCG-LH receptor complex is probably responsible for increased plasma progesterone secretion observed when ovine luteal cells are treated with hCG. Therefore, the longer half-life of hCG in the blood and slower turnover of the hCG-LH receptor complex on the surface of granulosa cells is likely responsible for an increased gonadotropic stimulation on the d 5 ovulatory follicle and its subsequent differentiation into a CL with a greater progesterone secreting capacity.

LH support is essential for development of a fully functional CL during metestrus (Burke et al., 1994; Peters et al., 1994). The persistence of hCG in plasma and on the cell surface may provide more optimal gonadotropic support for greater growth of the induced CL, that is formed from a follicle that has not been LH primed as is the case of a proestrus follicle at d 0. In contrast, treatment with a GnRH-agonist elevates plasma concentrations of LH for only 5 h. Furthermore, after d 5 of the estrous cycle, progesterone produced by the growing original CL has a negative effect on the frequency of LH pulses from the pituitary (Rahe et al., 1980; Savio et al., 1993). This would reduce original gonadotropic support necessary for functional development of the induced CL induced after ovulation of the first wave dominant follicle by GnRH-agonist. These two factors may explain the different progesterone secreting potentials of hCG and GnRH-agonist induced CL.

Progesterone content (µg/CL) of the induced CL before incubation was greater for the hCG induced CL. Since the hCG induced CL were heavier and there were no differences in the number of luteal cells (small and large) per surface area between T2 and T3, it can be concluded that the induced CL induced by hCG contained more luteal cells than the CL induced by the GnRH-agonist. During the process of differentiation of the follicular cells into luteal cells, granulosa cells differentiate into large luteal cells (Smith et al., 1994). No mitosis occurs during the differentiation of granulosa cells (Donaldson and Hansel, 1965; Zheng et al., 1994), and number of granulosa cells in the preovulatory follicle is the same as the number of large luteal cells after CL formation (Smith et al., 1994). However, intense mitotic activity is observed in theca-derived cells from d 1 to d 4 of the estrous cycle (Donaldson and Hansel, 1965; Zheng et al., 1994), and it is likely that theca cells differentiate into small luteal cells (Donaldson and Hansel, 1965; Alila and Hansel, 1984, Meidan et al., 1990).

It was suggested that small luteal cells also may originate from a population of stem cells composed of luteal fibroblasts (Niswender et al., 1985), and that during the estrous cycle small luteal cells may differentiate into large luteal cells. These processes are controlled by LH stimulation (Farin et al., 1988). Perhaps differentiation of fibroblasts into small luteal cells, transformation of small luteal cells into large luteal cells and increased mitogenic activity of the theca cells are all enhanced under increased LH-like stimulation due to hCG. This would explain augmentation of luteal weights observed for the hCG-treated animals, without a decrease in number of small and large luteal cells per surface area. It must also be noted that CL induced after administration of hCG (Fricke et al., 1993) or GnRH

(Webb, 1992) on d 6 of the estrous cycle do not differ in luteal weight from naturally occurring CL of the same age.

In vitro culture of the induced CL showed that both hCG or GnRH-agonist induced a functional CL. These results confirm those reported by Fricke et al. (1993) and Webb et al. (1992) for hCG and GnRH induced CL, respectively. However, LH stimulated progesterone production was greater for the hCG induced CL on a per g of tissue basis. Since only the small luteal cells have receptors for LH and respond to LH stimulation with an increase in progesterone production (Niswender et al., 1985), this may indicate an increased steroidogenic potential of the small luteal cells. In fact, morphometrical analysis of the luteal cells showed that small cells were larger for the hCG induced CL.

The characteristics of the small and large luteal cells were similar to those previously reported (Fields et al., 1985; 1991a; 1992; Weber et al., 1987). Stage 1 and 2 luteal cells were previously referred to as alpha and beta cells, respectively. Stage 1 are the most abundant cell type early in the estrous cycle (8-day old induced CL). Except for the smaller size and the presence of junctional complexes between adjacent cells, the small cells look like large cells. Several investigators have suggested that small cells develop into the large cells and that during the estrous cycle small luteal cells constantly replenish the pool of large luteal cells (Donaldson and Hansel, 1965; Cran, 1983; Alila and Hansel, 1984; Farin et al., 1988).

In the rat, the large luteal cell with its distended smooth endoplasmic reticulum and pleomorphic shaped nucleus contains more side chain cleaving enzyme, more sterol carrier protein 2, and is more steroidogenically active than the small cell with its oval nucleus and indistinct smooth endoplasmic reticulum (Fields, 1991a). Although nothing is known about the steroidogenic capabilities of the bovine luteal cell types at these various stages, the pleomorphic shape of the nucleus and distended smooth endoplasmic reticulum of the Stage 2 luteal cells morphologically resembles the large luteal cell of the rat. Stage 1 luteal cells with the oval shaped nucleus and indistinct smooth endoplasmic reticulum resemble the small luteal cell of the rat (Fields, 1991a). The results are congruent with an increase in plasma progesterone in the cow after d 8 of the estrous cycle, a time when the luteal cell population changes from being predominantly Stage 1 to Stage 2.

There is a higher percentage of Stage 1 and 2 luteal cells early in the estrous cycle (d 8 induced CL) and a high percentage of Stage 3 luteal cells later in the estrous cycle (d 17 untreated) The morphology of Stage 2 and 3 luteal cells is similar to those observed in luteal involution prior to regression reported in beef cattle (Fields, 1992). In beef cattle, luteal cells from 8-day old CL are healthiest as indicated by a peak in number of secretory granules and in secretion of oxytocin. Day 17 of the estrous cycle is a time when the CL is undergoing involution before regression as indicated by swollen mitochondria with a loss of their cristae and an accumulation of osmiophilic material (Fields et al., 1992). These results are consistent with the observation that, during the estrous cycle, Stage 2 and Stage 3 luteal cells, previously referred to as beta luteal cells, are more susceptible to subluteolytic doses of $PGF_{2\alpha}$ as indicated by their depletion of oxytocin containing secretory granules (Fields et al., 1991b). Under these conditions, secretory granules and mitochondria of the Stage 1, previously referred to as alpha luteal cells, were not affected. Stage 4 (regression) would be characterized by a large accumulation of lipid droplets, the complete loss of secretory granules, cell degeneration and the accumulation of collagen bundles among degenerating cells (Fields et al., 1992).

In Stage 3, the swelling of mitochondria and loss of cristae is likely due to an increased permeability of luteal cells which increased their sensitivity to changes in osmotic conditions during fixation. Properly fixed Stage 1 cells could be found adjacent to Stage 3 cells indicating proper fixation and embedding for a normal healthy cell.

In the original CL (d 17) of the hCG and GnRH-agonist treated cows, there was a larger percentage of Stage 1 and 2 luteal cells compared to the CL from untreated cows (d 17). These observations suggest that the additional LH-like exposure delayed progression of small and large luteal cells through the stages of development leading to the involution and regression processes. Perhaps extension of the CL lifespan, when cows are treated with LH during the second part of the estrous cycle is due to this effect of LH (Donaldson and Hansel, 1965b). This retardation in development of Stage 3 luteal cells of original CL treated with hCG and GnRH-agonist may be associated with several factors. The higher CL content of progesterone in unincubated tissue for hCG and GnRH-agonist treatment (Figure 5-3) indicated that CL involution was indeed retarded. Diaz et al. (1993) demonstrated that cattle treated with hCG on d 5, all had three wave follicle cycles and a slightly longer estrous cycle. Therefore, a delay in luteolytic signaling (e.g. estradiol and $PGF_{2\alpha}$) may have occurred and this is reflected by the lower frequency of Stage 3 luteal cells in hCG and GnRH-agonist treated cows.

No major differences in morphological characteristics of luteal cells (e.g., distribution of Stage 1 and Stage 2) were detected between hCG and GnRH induced CL. Thus, the increased capacity of hCG induced CL to secrete progesterone (in vivo and in vitro) is related to increased number of luteal cells associated with a greater tissue weight and a greater sensitivity to LH that is not reflected by the distribution of Stage 2 and Stage 3 luteal cells (small or large). Perhaps a greater number of LH receptors resides on the small luteal cells of hCG induced CL. However, none of the experimental approach examined addressed this possibility. In cattle, progesterone secretion by the large luteal cells is not increased with LH stimulation (Hansel et al., 1991). The role of the LH receptors identified on bovine large luteal cells (Chegini et al., 1991), may be to prevent progression of the cells through stages of development leading to regression of the CL.

In vitro incubation did not detect any treatment differences (hCG vs GnRH-agonist) in basal or LH stimulated progesterone production of luteal slices of the naturally occurring CL (µg progesterone / g CL) removed on d 17. Furthermore, no differences were detected in progesterone secretion per CL. Comparable rates of in vitro progesterone secretion by original CL would account for the similar plasma concentrations of progesterone in vivo after the induced CL were removed. However, before incubation the original CL in GnRH-agonist and hCG treated animals contained more progesterone than that of the control group. Furthermore, size of the small and large luteal cells were increased for the animals treated with hCG or the GnRH-agonist. This is consistent with other reports in ewes (Farin et al., 1988) where size of luteal cells of the original CL is increased after treatment with pharmacological doses of hCG or LH. This may be indicative of healthier (Stage 1) luteal cells.

Implications

Ovulation of the first wave dominant follicle on d 5 of the estrous cycle after administration of a GnRH-agonist or hCG induced a functional induced CL responsible for the subsequent increases in plasma progesterone. The extended LH-like stimulation due to the injection of hCG produced a larger and more steroidogenic CL. Development of new pharmacological forms and delivery systems of GnRH to extend the release period of LH to replace hCG, a potentially immunogenic molecule, warrants investigation. Of interest is the observation that luteal cells progress sequentially through three morphological stages before regression. This process could be delayed by the action of LH.

CHAPTER 6

INFLUENCE OF LH PRIMING BEFORE OVULATORY INDUCTION WITH A GnRH-AGONIST OF THE FIRST WAVE DOMINANT FOLLICLE AND SUBSEQUENT CORPUS LUTEUM FUNCTION

Introduction

Experimental evidence is limited in cattle as to whether quality of the preovulatory follicle is associated with subsequent function of the corpus luteum (CL). Pulsatile secretion of luteinizing hormone (LH) during the luteal phase of the estrous cycle follows an episodic pattern of high amplitude and low frequency pulses that account for a low basal secretion rate of LH (Rahe et al., 1980; Walters and Schallenberger, 1984; Cupp et al., 1995). This reduced secretion of LH is a consequence of high progesterone secretion by the CL that causes turnover of the dominant follicle and initiation of a new follicular wave (Sirois and Fortune, 1990; Savio et al., 1993b). Variations in the pattern of LH secretion as a consequence of variable progesterone and progestin concentrations have been characterized (Roberson et al., 1989; Savio et al., 1993b; Bergfeld et al., 1995). In a low progesterone environment (proestrous phase), frequency of LH pulses is high and amplitude of pulses is low, whereas in a high progesterone environment (luteal phase), frequency of LH pulses is low and amplitude high (Rahe et al., 1980;

Roberson et al. 1989; Kawate et al., 1993; Cupp et al., 1995). These natural conditions can be simulated with artificial progestins such as norgestomet. For instance, treatment of heifers with four implants of norgestomet (6 mg, 1 implant) induces a pattern of LH secretion characteristic of the natural mid-luteal phase and causes a regular turnover of the dominant follicle (Sanchez et al., 1995). Treatment with one norgestomet implant induces a pattern of LH secretion similar to what is observed during the proestrous phase after regression of the CL. This endocrine state prevents atresia of the dominant follicle that will continue to grow and secrete estradiol (Savio et al., 1993b; Sanchez et al., 1995).

During the proestrous phase of the estrous cycle, following regression of the CL and the decline in plasma progesterone, there is a linear increase in mean concentration of LH before the pre-ovulatory surge (Chenault et al., 1975; 1976; Imakawa et al., 1986). During this period, frequency of LH pulses increases under the stimulatory effect of estradiol secreted by the pre-ovulatory follicle and ultimately leads to induction of the pre-ovulatory surge of LH (Chenault et al., 1975; 1976; Imakawa et al., 1986, Stumpf et al., 1989).

Growth and differentiation of the ovulatory follicle during the follicular phase is essential to formation of a functional CL (McNatty, 1979). In ewes, when follicles are induced to ovulate with an injection of GnRH during a period of low LH secretion (seasonal anestrus), normal luteal function does not occur (Haresign et al., 1975). Several reports indicate that the change in pattern of LH secretion before the pre-ovulatory surge of LH is essential for follicular maturation as judged by the function of the subsequent CL (McNatty et al., 1981; Baird, 1992). Such a relationship has not been demonstrated in cattle. In an experiment designed to investigate the relationship between pre-ovulatory and post-ovulatory gonadotropin secretion in post-partum beef cattle, no differences in gonadotropin secretion were detected between animals expressing short or normal luteal phases (Garverick et al., 1988). Subsequent studies demonstrated that short luteal phases observed in post-partum beef cattle are due to an early release of $PGF_{2\alpha}$ (Garverick et al., 1992).

In cattle the first wave dominant follicle will ovulate after an injection of $PGF_{2\alpha}$ between d 5 to d 8 after estrus, when the first wave dominant follicle is in a growing phase or early plateau phase (Kastelic et al., 1990; Savio et al., 1990). Ovulation will not occur when luteolysis is induced at a time when the first wave dominant follicle is at the end of its plateau phase and a second wave dominant follicle has been identified (Kastelic et al., 1990).

Characteristics of an ovulatory follicle can be studied by inducing luteolysis with $PGF_{2\alpha}$ on d 5 after estrus and concurrently inserting a progestin implant that will induce low concentrations of circulating progestins (Sirois and Fortune, 1990; Savio et al., 1993a, 1993b; De la Sota et al., 1993; Cupp et al., 1993). In a low progestin environment, the first wave dominant follicle will continue its growth and escape atresia. When removed on d 10 (Cupp et al., 1993) or on d 12 (De la Sota et al., 1993), this sustained first wave dominant follicle contains high follicular fluid concentrations of androstendione and estradiol, high concentrations of estradiol

(De la Sota et al., 1993), and both granulosa and thecal cells contain an increased number of LH receptors (Cupp et al., 1993).

Objectives of the present experiments were: 1) to determine if a first wave dominant follicle maintained in a high or low progestin environment will ovulate after a GnRH-agonist injection given on d 8.5 of the estrous cycle; 2) to evaluate importance of LH priming of the first wave dominant follicle on subsequent function of the CL when the follicle is induced to ovulate on d 8.5 of the estrous cycle.

Materials and Methods

Previous studies indicated that a pattern of LH release characteristic of diestrus can be induced in heifers treated on d 5 of the estrous cycle with an injection of $PGF_{2\alpha}$ and insertion of four norgestomet implants (Sanchez et al., 1995). If only one implant is administered then secretion of LH mimics the pattern observed during the follicular phase (Savio et al., 1993b; Sanchez et al., 1995). Furthermore, administration of GnRH can induce ovulation of the first wave dominant follicle and formation of an accessory CL when treatment is given on d 5 (Schmitt et al., 1993) or d 6 (Rusbridge et al., 1992) of the estrous cycle. Thus, with these experimental means to manipulate LH secretion and to induce ovulation, the following experiments were designed.

Experiment 1

Nonlactating Holstein cows (n = 25) were kept in a lot with minimal pasture and fed peanut hay and corn silage. Estrus was synchronized with s.c. insertion of a norgestomet ear-implant for 7 d (Syncro-Mate B; 6 mg of norgestomet implant used without the injection of the norgestomet/estradiol valerate solution; Sanofi Animal Health, Overland Park, KS) and an injection of $PGF_{2\alpha}$ (Lutalyse ; 25 mg i.m.; The Upjohn Company, Kalamazoo, MI) at 24 h before implant removal. Cows were observed for estrus twice daily (0600 to 0700 and 1800 to 1900) following removal of the implant. Tail heads were painted (Impervo, Benjamin Moore and Co., Montvalo, NJ) and chalked (All-weather Paintstick, LA-CO Industries, Chicago, IL) as an aid for estrus detection (Macmillan et al., 1988). Ten cows observed in estrus on the same day (d 0) were assigned randomly to two treatment groups (Figure 6-1).

In the first group (1 norgestomet implant = 1Ni), cows received one norgestomet ear-implant on d 5.5 after estrus and two injections of $PGF_{2\alpha}$ (Lutalyse, 25 mg i.m.) on d 5.5 and d 6. This treatment sequence was designed to induce a low progestin environment, increase the frequency of LH pulsatility, and stimulate continuous growth of the first-wave dominant follicle (Taylor et al., 1993; Savio et al., 1993b).

In the second group (5 norgestomet implants = 5Ni) cows received five norgestomet ear-implants on d 5 after estrus and two injections of $PGF_{2\alpha}$ (Lutalyse, 25 mg i.m.) on d 5.5 and d 6. This treatment sequence was to mimic a high progestin environment, to induce a pattern of low LH pulsatility similar to what is observed during the mid luteal phase (Sanchez et al., 1995). Furthermore, norgestomet does not cross-react with the antibody used in the radioimmunoassay for progesterone (unpublished observations). This permitted a monitoring of CL status during the experiment.

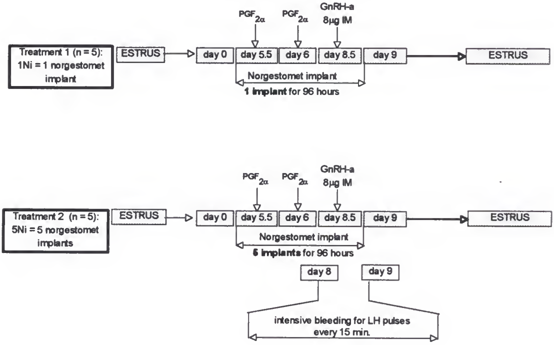


Figure 6-1. Treatment sequence for ovulation of a first wave dominant follicle maintained in a low (1Ni) or (5Ni) high progestin environment with a GnRH-agonist injection.

On d 8.5, all cows received an injection of a GnRH-agonist (Buserelin, 8 µg i.m.; Hoechst-Roussel Agri-Vet, Somerville, NJ) to induce a pre-ovulatory surge of LH. On d 9, the norgestomet implants were removed.

Ovaries in each cow were examined transrectally on a daily basis with an Equisonic LS 1000 linear-array ultrasound device equipped with a 7.5 megaHertz transducer (Tokyo Keiki Co., Tokyo, Japan) to assess regression of the CL, presence of the first wave dominant follicle, ovulation of the first wave dominant follicle and formation of the subsequent CL.

Blood (10 ml) samples were collected daily by jugular venipuncture into evacuated heparinized tubes (Vacutainer®, Becton Dickinson, Rutherford, NJ) and immediately centrifuged (3,000 x *g* for 20 min). On d 8, a gas-sterilized polyvinyl jugular catheter (BB317-V10, i.d. 1.57 mm, o.d. 2.8 mm, Bolab, Lake Havasu City, AZ) was inserted into the jugular vein of each experimental cow. Ten ml of blood were collected every 15 min for 7 h before and 7 h after administration of the GnRH-agonist. This intensive blood sampling was implemented to monitor the secretory pattern of LH before and after injection of the GnRH-agonist.

Experiment 2

The second experiment was a replicate of the first experiment with ten cows (Figure 6-2). However, implants of norgestomet removed on d 9 were replaced by five new norgestomet implants for all the experimental cows. The newly inserted norgestomet implants were removed on d 18 of the experimental sequence. Insertion of five implants was performed in an attempt to avoid premature luteal regression due to early secretion of $PGF_{2\alpha}$. Blood sampling and ultrasonography were performed as described previously but the intensive sampling for LH of the first experiment was not repeated.

Both experiments, were designed to provide differential LH priming of the pre-ovulatory follicle prior to injection of the GnRH-agonist.

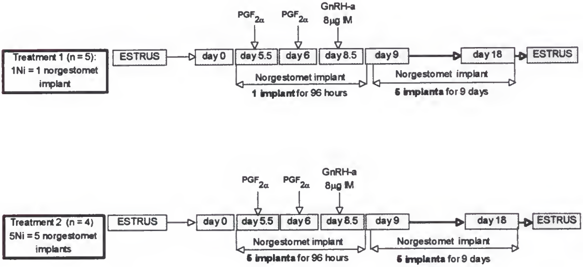


Figure 6-2. Treatment sequence for a first wave dominant follicle maintained in a low (1Ni) or high (5Ni) progestin environment before ovulation with a GnRH-agonist injection. On d 9 of the experimental sequence 5 norgestomet implants were administered to all the experimental cows and removed on d 18.

Radioimmunoassay (RIA)

Plasma was separated and stored at -20°C until it was assayed for progesterone (Knickerbocker et al., 1986) and estradiol (Badinga et al., 1992). Plasma concentrations of progesterone and estradiol were measured with single-antibody radioimmunoassays. Sensitivity of the estradiol assay was .65 pg/ml. Inter and intra-assay coefficients of variation (CV) were 1.35 % and 7.35 %. The minimum detectable concentration of progesterone was .15 ng/ml. Intra-assay and inter-assay CV were 8.78 % and 10.5 %. Plasma concentrations of LH were determined with a double antibody RIA as described previously (Lucy et al., 1991). The limit of detection of the assay, expressed as ng USDA-bLH-B6/ml, was 0.05 ng/ml. Intra-assay and inter-assay coefficients of variation were 10.5 % and 8.4 %.

Statistical Analyses

Data were analyzed using the General Linear Models Procedure of the Statistical Analysis System (SAS, 1988). Plasma concentrations of progesterone and estradiol were analyzed using a model that examined the effects of treatment, cow(treatment), time of sampling and the interactions treatment x time and cow(treatment) x time. Cow was considered as a random effect.

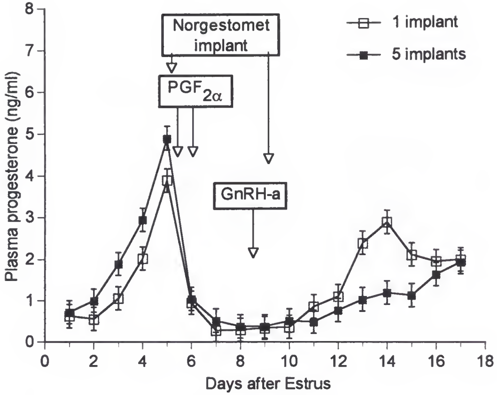
LH pulsatile patterns of individual cows were analyzed by the Cluster pulse analysis algorithm (Veldhuis et al., 1985) for evidence of significant LH secretory events (LH peaks). For the application of the cluster program relative to decision analysis, each peak was defined as an increase in LH that exceeded the preceding LH value by the mean LH concentration for the cow plus one standard deviation. These criteria and definition were reported by Bishop and Wettemann (1993). Pulse amplitude was the difference between the greatest value during a pulse and the nadir within 30 min before the pulse. Number of peaks during the sampling period and amplitude of peaks were analyzed with the General Linear Model procedure of SAS (1988) using mathematical models that included the effect of treatment for the number of LH peaks, and treatment, cow(treatment) for analysis of LH peak amplitude.

Results

Experiment 1

At the onset of the experimental sequence, administration of $PGF_{2\alpha}$ induced a rapid decline in plasma progesterone associated with luteolysis in both treatment groups (Figure 6-3). This decline in progesterone was followed by regression of the corpus luteum in which the corpus luteum could not be detected by ultrasonography on d 8.8 ± .4 for 1Ni and on d 9.4 ± .8 for 5Ni.

The progestin treatment induced a difference in LH pulsatility between the two treatment groups. Luteinizing hormone pulse frequency (number of pulses /h;



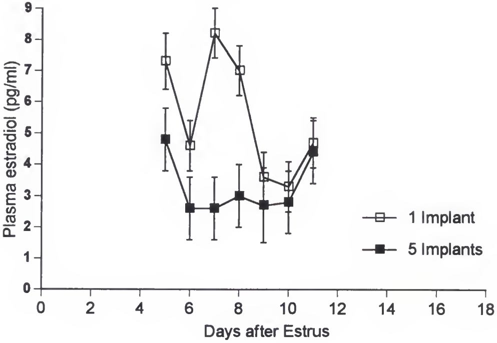


Figure 6-3. Plasma concentrations (mean ± SEM) of progesterone (top panel) and estradiol (bottom panel) for Experiment 1

Figure 6-4, Table 6-1) was increased for 1Ni (0.63 ± .06) when compared to 5Ni (0.29 ± .1; *P* < .05). One experimental cow (#2001) of the 5Ni group did not respond to the treatment and presented a high frequency of LH pulses (4 pulses, Figure 6-4). This difference could not be explained after visual inspection of follicular growth pattern or the plasma estradiol profile for cow # 2001. LH pulse amplitudes or LH means for the first 7 h of the sampling period (ng/ml) did not differ between 1Ni and 5Ni treatments when the data set was analyzed with or without cow # 2001.

Table 6-1. Characteristics (means ± SEM) of LH secretion during 7 h of intensive blood sampling before injection of the GnRH-agonist.

	One implant	Five implants
Number of cows	5	5
LH pulses / h *	0.63 ± .06	0.29 ± .1
LH peak amplitude (ng/ml)	0.28 ± .03	0.32 ± .05
LH mean (ng/ml)	0.25 ± .04	0.19 ± .04

* 1Ni > 5Ni: *P* < .05

Associated with the higher LH pulse frequency of cows treated with one norgestomet implant (Table 6-1) was a higher concentration of plasma estradiol (Figure 6-3) from d 6 to d 9 (5.9 ± .7 pg/ml) compared to cows treated with five

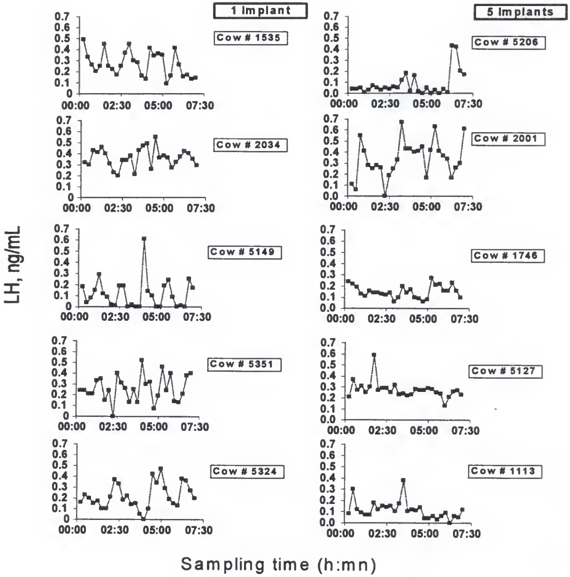


Figure 6-4. Individual LH secretory patterns of cows treated with 1 norgestomet implant (left panel) or 5 norgestomet implants (right panel) before a GnRH-agonist ovulatory challenge. Intensive blood sampling was carried on for 7 h before administration of the GnRH-agonist.

norgestomet implants (2.7 ± .9; P<.03). This implies that a more functional follicle developed in the 1Ni group. Indeed, average size of the first wave dominant follicle was greater (P < .001) in the 1Ni group (14.6 ± .6 mm) compared to the 5Ni (12.3 ± .6 mm) group from d 6 to d 9 of the experimental sequence (Figure 6-5).

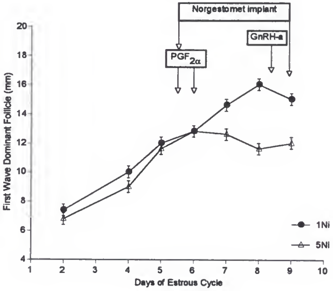


Figure 6-5. Size of the first wave dominant follicle (mean ± SEM) from d 2 of the estrous cycle until ovulation for cows treated with 1 norgestomet implant (1Ni) or 5 norgestomet implants after luteolysis induced by $PGF_{2\alpha}$ on d 5.5 of the estrous cycle for the first experiment.

Administration of a GnRH-agonist on d 8.5 of the estrous cycle induced a greater release of LH (Figure 6-6) in the 1Ni group (P < .001) that had a higher plasma estradiol concentration. Mean plasma concentrations of LH (ng/ml) during the 7 h after injection of the GnRH-agonist (Figure 6-6) were significantly increased for 1Ni (5.8 ± .6) compared to 5Ni (3.8 ± .6; P < .05). This difference was due to a longer duration of the surge (5 versus 6.5 h) and a larger peak amplitude (1Ni = 15.2 ± .6 ng/ml > 5Ni = 10.5 ± .6 ng / ml; *P* < .001).

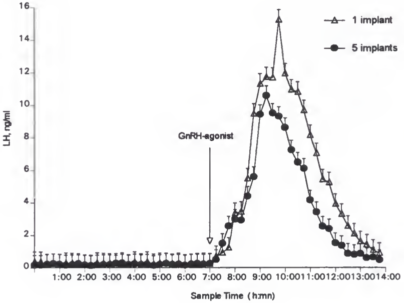


Figure 6-6. Plasma concentrations of LH (mean ± SEM) during a 14 intensive blood sampling period. Blood was sampled every 15 min and a GnRH-agonist administered 7 h after the beginning of the sampling

Administration of the GnRH-agonist induced a surge of LH that was sufficient to cause ovulation of the first wave dominant follicle in all five cows of 1Ni and four of five cows of 5Ni (*P*<.29). Ovulation occurred on d 10 of the treatment sequence, and a subsequent CL was formed as verified by ultrasonography. Plasma concentrations of progesterone (Figure 6-3; ng/ml) differed between the treatment groups between d 10 to d 14 [1Ni = 1.5 ± 0.2] > [5Ni = 0.8 ± 0.3]; *P* < 0.06). An early decline of progesterone concentrations was due to the short lifespan of the induced CL (11.5 ± 2.2 d; difference between the time when the CL is first detected by ultrasonography and the time when the luteal structure is no longer visible).

Premature regression of the growing CL could be due to a release of $PGF_{2\alpha}$ from the endometrium. Secretion of estradiol from growing follicles can induce the release of $PGF_{2\alpha}$ (Silvia et al., 1991). Indeed, on d 11 of the experimental sequence, plasma concentrations of estradiol in both treatment groups were increased and may have triggered early luteolysis. It was hypothesized that inserting five new norgestomet implants 12 h after the injection of GnRH-agonist would mimic luteal phase concentrations of progesterone and prevent growth of estrogenic follicles (Sanchez et al., 1995). This strategy was adopted in the second experiment to allow for better expression of the steroidogenic potential of the CL induced by administration of the GnRH-agonist.

Experiment 2

As observed in the first experiment, administration of $PGF_{2\alpha}$ induced a rapid decline in plasma progesterone associated with luteolysis (Figure 6-7). The decline in plasma concentrations of progesterone was followed by regression of the corpus luteum on d 8.8 ± .4 for all cows in the 1Ni group and on d 8.6 ± .4 for cows of the 5Ni group. In the 5Ni group, one cow did not regress the CL after injections of $PGF_{2\alpha}$ and was removed from the experiment.

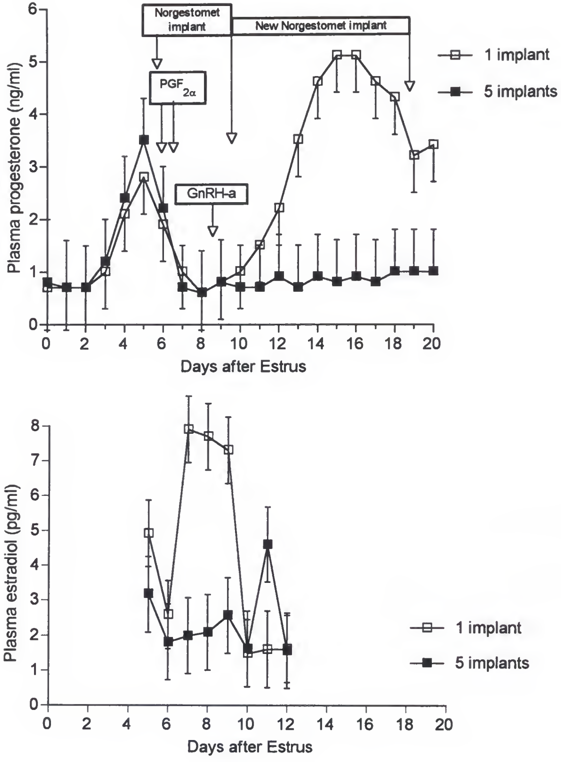


Figure 6-7. Plasma concentrations (mean ± SEM) of progesterone (top panel) and estradiol (bottom panel) for Experiment 2.

Based on the higher plasma concentrations of estradiol (Figure 6-7) between d 6 to d 9 (6.4 ± .4 pg/ml) compared to cows treated with 5 norgestomet implants (2.1 ± .4 pg/ml; $P<.001$), cows treated with one norgestomet implant appeared to have a high frequency of LH pulsatility as observed in the first experimental sequence,.

Administration of the GnRH-agonist provoked ovulation in five cows of the 1Ni group. Ovulation occurred on d 10.6 ± .2. However, only one animal of the 5Ni group (one of four cows) ovulated in response to the GnRH-agonist on d 11 ($P<.01$). This low ovulatory (5Ni < 1Ni) response probably was caused by insertion of five new norgestomet implants at 12 h after injection of the GnRH-agonist.

All cows that ovulated formed a CL as assessed by ultrasonography. Following treatment with a GnRH-agonist, plasma concentrations of progesterone (ng/ml) were increased significantly from d 10 to d 18 of the experimental sequence in the 1Ni group (3.3 ± .2) as compared to the 5Ni group (0.8 ± .2; $P<.001$; Figure 6-7). Immediately after removal of the norgestomet implants on d 18, the induced CL regressed on d 20 ± .7 for four cows in the 1Ni group and on d 19 for the cow in the 5Ni group. One cow in the 1Ni group had not regressed the GnRH-agonist induced CL by d 35, when the experiment was terminated. This cow had a higher concentration of progesterone than all other cows in the 1Ni treatment group on d 20 (11.24 > 1.32 ± .5, ng / ml) and was responsible for the elevated plasma concentrations of progesterone on d 20 (Figure 6-7). When the data set was analyzed without this latter cow, plasma concentrations of progesterone were higher in 1Ni than in 5Ni cows from d 10 to d 20(*P* <.001).

Insertion of five norgestomet implants at 12 hours after administration of the GnRH-agonist allowed a better expression of the steroidogenic potential of the GnRH-agonist induced CL in the 1Ni treatment group (Figure 6-7). However, lifespan of the subsequent luteal structures was lengthened in only one experimental animal. Furthermore, this treatment prevented ovulation of the first wave dominant follicle in the 5Ni group.

Discussion

In the first experiment, most of the first wave dominant follicles ovulated after injection of the GnRH-agonist. Other investigators observed that when luteolysis is induced on d 7 or d 8 of the estrous cycle the first wave dominant follicle will ovulate (Savio et al., 1990; Kastelic et al., 1990). In the 1Ni group, the first wave dominant follicle, maintained in a low progestin environment, was very estrogenic due to increased LH support as reported previously (Savio et al., 1993b; De la Sota et al., 1993; Cupp et al., 1993). This indicates that estrogenicity of the first wave dominant follicle does not necessarily have a direct effect on its capacity to ovulate since most of the follicles in both groups ovulated after administration of the GnRH-agonist in experiment 1.

In the second experiment, only one first wave dominant follicle in the 5Ni group responded to the injection of GnRH-agonist and ovulated. This suggests a need for LH support after the induced pre-ovulatory surge of LH to achieve ovulation in follicles that were not primed sufficiently with LH prior to injection of the GnRH-agonist injection. Perhaps insertion of five implants at 12 h after injection of the GnRH-agonist diminished basal LH secretion and inhibited final maturation of the follicle after the pre-ovulatory surge of LH. This appears to be the case because in experiment 1, without re-insertion of 5 implants, 4 of 5 cows ovulated. Alternatively, high amounts of norgestomet may have a direct effect on responsiveness or maturation of the follicle. The block to ovulation with high levels of progestogens at the end of the pre-ovulatory surge of LH may define a new physiological and sensitive window in the ovulatory process. Thus, the pre-ovulatory surge of LH may not be the sole and ultimate gonadotrophic requirement for ovulation. This sensitivity to high progestins may be over exaggerated in the absence of correct LH priming of the pre-ovulatory follicle.

The short luteal phase observed in both groups of the first experiment could be due to inadequate gonadotropic support of the ovulatory follicle or an early release of $PGF_{2\alpha}$ from the uterus. The pre-ovulatory LH surges, induced by the GnRH-agonist that ovulated the follicle in the two treatment groups, were not comparable to those observed during the follicular phase of a normal estrous cycle (Chenault et al., 1975, 1976; Rahe et al., 1980). In both treatment groups, the induced pre-ovulatory surge of LH was shorter (5 to 6.5 h) compared to what was observed during a naturally occurring estrus (10 to 12 h). Furthermore, the frequency of LH pulsatility during the period preceding the pre-ovulatory surge of LH (0.63 ± .06 pulses / h in 1Ni group; 0.29 ± .1 pulses / h in 5Ni group) was lower than what has been recorded during a normal estrous cycle (1.5 pulse / h, Walters and Schallenberger, 1984). Although the induced pre-ovulatory surge was greater in treatment one, this group still expressed a short cycle.

A plausible explanation for early regression of the CL could be a premature release of $PGF_{2\alpha}$. When the endometrium of cows have been exposed to a progestin for more than 12 d, the "progesterone block" ends in association with a decline in uterine progesterone receptors. This is followed by a passive increase in uterine oxytocin receptors or an increase in oxytocin receptors in response to the action of estradiol from active follicles that induced estradiol receptors and transcription of oxytocin receptors (McCracken et al., 1984; Meyer et al., 1986). The increase in endometrial oxytocin receptors initiates production of luteolytic pulses of $PGF_{2\alpha}$ (McCracken et al., 1984).

The rationale for experiment 2 was that insertion of five norgestomet implants in all treated cows would reduce gonadotrophic support to the GnRH-agonist recruited follicle (Macmillan and Thatcher, 1991), reduce secretion of estradiol that otherwise would stimulate up-regulation of oxytocin receptors and initiate uterine secretion of $PGF_{2\alpha}$ pulses. In addition, five implants would exert a progestin block. This modification did not greatly extend lifespan of the CL as measured by ultrasonography. However, the plasma concentrations of progesterone did not decrease appreciably until after removal of the implants. Perhaps the norgestomet implants effectively antagonized an early release of $PGF_{2\alpha}$ from the endometrium thereby delaying functional steroidogenic regression of the CL.

In experiment 1 differences in luteal phase progesterone concentration between 1Ni and 5Ni were probably due to a combination of two factors: an increased frequency of LH pulsatility before the pre-ovulatory surge of LH between the two treatments, and a more robust induced pre-ovulatory surge of LH for the 1Ni group. This demonstrates the importance of LH priming of the follicle before ovulation. Without induction of follicular estradiol secretion as a consequence of increased LH pulsatility, a greater induced surge of LH would not have occurred. The greater priming with increased LH pulses and induction of estradiol most likely increased LH receptors on granulosa cells (Richards, 1980, Kessel et al., 1985). This could have increased subsequent luteinization and differentiation of luteal cells in response to the GnRH induced surge of LH. Collectively, these mechanisms would enhance subsequent luteal phases of the 1Ni group that was observed in both experiments (1 and 2). Similar results were obtained in sheep where plasma concentrations of LH preceding the pre-ovulatory surge of LH were an important determinant of follicular maturation as judged by subsequent plasma concentration of progesterone (McNatty et al., 1981; Baird, 1992).

In the second experiment, inserting five norgestomet implants 6 hours after the pre-ovulatory surge of LH may have reduced LH support of the growing CL and hastened its demise (Peters et al., 1995). Burke et al. (1995) prematurely induced elevated concentrations of progesterone with insertion of an intravaginal progesterone releasing device on d 1 of the estrous cycle. This treatment induced formation of a CL with a reduced lifespan but did not prevent ovulation.

The increased release of LH after injection of the GnRH-agonist in the 1Ni group of experiment one is probably due to higher concentrations of estradiol observed after induced regression of the CL. Luteinizing hormone response to GnRH is increased in cattle during periods of increased concentrations of estradiol in the blood (Zolman et al., 1974; Thatcher and Chenault, 1976). Stumpf et al. (1989, 1991) reported that increased levels of estradiol are required to initiate the pre-ovulatory surge of LH in cattle and that estradiol increases the amplitude of the pulses of LH. Furthermore, in studies examing effect of gonadal steroids on LH release from bovine pituitary cells cultured in vitro, basal and GnRH-stimulated release of LH was enhanced by exposure to estradiol (Padmanabhan et al., 1978; Baratta et al., 1994).

Implications

This study suggests that an increased LH pulse frequency before the pre-ovulatory surge of LH is an important determinant of follicular maturation and subsequent function of the CL. Further studies are warranted to precisely isolate the specific role of follicular maturation before ovulation to optimize estrus synchronization protocols that permit a timed-insemination. The potential ovulatory follicle appears to be sensitive to gonadotropin support both prior to and after the pre-ovulatory surge of LH relative to subsequent CL performance.

CHAPTER 7
USE OF A GnRH-AGONIST OR hCG FOR TIMED-INSEMINATION IN CATTLE

Introduction

Estrus synchronization is essential for implementation of artificial insemination (AI) in management situations where estrus detection can be difficult, as with large dairy herds, post partum cows, cows that are heat stressed and breeds exhibiting estrus for a reduced amount of time (Narasimha Rao and Venkatramiah, 1991; Chenault, 1992). Furthermore reproductive efficiency is the product of estrus detection and conception rates (Macmillan, 1992). Thus, maximizing estrus detection rates can improve overall pregnancy rates. Several systems have been described for synchronization of estrus in cattle (Odde, 1990). Most of the protocols synchronize estrus by manipulating the plasma concentration of progesterone. Injection of $PGF_{2\alpha}$ induces regression of the corpus luteum (CL) allowing growth and ovulation of a follicle. In contrast, progesterone-releasing devices prevent a preovulatory surge of LH by maintaining progestogen exposure until the CL regresses with subsequent development of the ovulatory follicle. Since estrus is the consequence of coordinated growth of a follicle and increased estradiol secretion coupled with demise of the CL (Chenault et al., 1975 and 1976), regulation of follicular growth and estradiol secretion, and secretion of progesterone by the CL may provide a means to enhance precision of estrus synchronization (Fogwell et al., 1986). Injecting estradiol valerate at the beginning of a progestogen treatment, i.e. the Syncromate-B synchronization system (Odde, 1990), programs follicular development (Adams, 1994; Bo et al., 1995).

Recently, use of a GnRH-agonist injection followed 7 d later by an injection of $PGF_{2\alpha}$ (Thatcher et al., 1989; Coleman et al., 1991; Twagiramungu et al., 1992; Wolfenson et al., 1994) has been used effectively to synchronize estrus. With this protocol, control of follicular growth and CL regression allows for precise synchronization of estrus (Twagiramungu et al., 1992; Thatcher et al., 1993). Synchronization of follicular growth contributed to greater precision in timing of estrous behavior (Thatcher et al., 1989; Wolfenson et al., 1994) when compared to synchronization protocols based on the use of $PGF_{2\alpha}$ alone to control regression of the CL. More than 90% of the detected estruses occurred during the 4 d following injection of $PGF_{2\alpha}$ when GnRH-agonist was administered 7 d before $PGF_{2\alpha}$. Furthermore, the dominant preovulatory follicle can be induced to ovulate by injecting a GnRH-agonist. Ovulation occurred 30 h after the GnRH-induced surge of LH (Roche, 1975; Pursley et al., 1994b; Silcox et al., 1995). Inducing a surge of LH after synchronization of follicle growth and CL regression should program ovulation and enable AI at a fixed time, eliminating the need for estrus detection.

The objective of this series of experiments was to establish a sequential pharmacological treatment that would allow AI at a fixed time with normal fertility. Such a management program would eliminate the need for estrus detection.

Materials and Methods

Three experiments were conducted at the heifer unit of a large commercial dairy in southern Florida (Larson's Dairy, Okeechobee, FL). Holstein heifers, 14 to 17 mo old, weighing 350 ± 40 kg, were used in this study. Heifers were maintained on natural pasture and fed 3 kg of concentrate per animal per day. Heifers were observed for signs of behavioral estrus twice daily (0600 h and 1800 h) for 90 min. A modified tail-head chalk system was implemented to aid in detection of estrus (Macmillan et al., 1988). All heifers were bred with frozen/thawed semen from five bulls with good fertility. Inseminations were performed by the same technician, once a day, after the morning detection of estrus (0730 h). Heifers therefore were inseminated 0 to 12 h after estrus detection and 0 to 24 h after beginning of estrous behavior.

Experiment 1: GnRH at 24 h After $PGF_{2\alpha}$

The first field study was designed to test the efficacy of using timed-insemination after an injection of GnRH-agonist 24 h following administration of

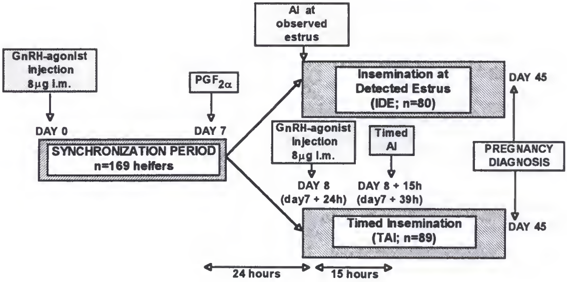

AI at observed estrus
GnRH-agonist injection 8μg i.m.
PGF$_{2\alpha}$
Insemination at Detected Estrus (IDE; n=80)
DAY 45
DAY 0
DAY 7
SYNCHRONIZATION PERIOD n=169 heifers
GnRH-agonist injection 8μg i.m.
Timed AI
PREGNANCY DIAGNOSIS
DAY 8 (day7 + 24h)
DAY 8 + 15h (day7 + 39h)
Timed Insemination (TAI; n=89)
DAY 45
24 hours
15 hours

Figure 7-1. Experimental design for the first timed-insemination experiment.

PGF$_{2\alpha}$. For this experiment, heifers were allocated randomly to two treatment groups: timed artificial insemination (TAI; n=89) heifers were inseminated at a predetermined time, and insemination at detected estrus (IDE; n=80) heifers were inseminated after observation of a standing estrus. Heifers (n=169) were synchronized with GnRH-agonist (Buserelin, 8 μg i.m.; Hoechst -Roussel Agri-vet Sommerville, NJ) administered on Day 0 of the experimental sequence (treatments were initiated independently of the stage of the estrous cycle) at 1700 h . This injection ovulates or luteinizes any mature follicle present on the ovary at the time of treatment, and recruits a cohort of new follicles one of which becomes the dominant follicle 7 d later (Macmillan and Thatcher, 1991; Wolfenson et al., 1994). The $PGF_{2\alpha}$ (Lutalyse®, 25 mg i.m.; The Upjohn Company, Kalamazoo MI) was administered on Day 7 (1700 h) to regress a spontaneous or Buserelin induced CL. Heifers in the TAI system received a second administration of GnRH-agonist 24 h after the injection of $PGF_{2\alpha}$ (Day 8, 1700 h; Figure 7-1). The purpose of the second GnRH-agonist injection is to induce a preovulatory surge of LH that will ovulate the dominant follicle recruited by the first injection of Buserelin. Previous studies showed that ovulation occurs 30 h after the induced surge of LH (Roche, 1975; Pursley et al, 1994b; Silcox et al., 1995). Heifers receiving TAI were inseminated 15 h after the GnRH-agonist injection (Day 9, 0800 h) to allow time for sperm capacitation. Heifers receiving IDE were inseminated 0 to 12 h after observation of standing estrus. Heifers were diagnosed for pregnancy by palpation per rectum 45 d after insemination.

Experiment 2: GnRH at 48 h After $PGF_{2\alpha}$

The second field study was designed to test the efficacy of using a timed-insemination following injection of GnRH-agonist 48 h after administration of $PGF_{2\alpha}$. Two replicates of this experiment were conducted. Heifers in the first replicate (TAI, n=93; IDE, n=88) and in the second replicate (TAI, n=94; IDE, n=89) were managed similarly (Figure 7-2). No animals of the first replicate were used in the second replicate. The synchronization protocol was identical to the protocol used for Experiment 1 (GnRH-agonist and $PGF_{2\alpha}$ given 7 d apart) except that the second injection of GnRH-agonist for the TAI group was given 48 h after the injection of $PGF_{2\alpha}$ (Day 9 at 1700 h). Delaying the GnRH-agonist injection by 24 h in the TAI group potentially increased the time between CL regression and ovulation. This would allow a longer period of elevated basal and increased pulsatility of LH (Rahe et al., 1980) and therefore improve the gonadotrophic priming of the ovulatory follicle. Such a stimulated ovulatory follicle may

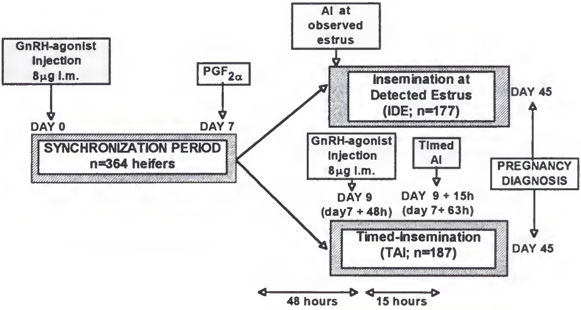

GnRH-agonist injection 8μg i.m.
PGF2α
DAY 0
DAY 7
SYNCHRONIZATION PERIOD n=364 heifers
AI at observed estrus
Insemination at Detected Estrus (IDE; n=177)
DAY 45
GnRH-agonist injection 8μg i.m.
Timed AI
DAY 9 (day7 + 48h)
DAY 9 + 15h (day 7+ 63h)
PREGNANCY DIAGNOSIS
Timed-Insemination (TAI; n=187)
DAY 45
48 hours
15 hours

Figure 7-2. Experimental design for the second timed-insemination experiment.

differentiate into a CL with improved steroidogenicity and lifespan and potentially reduce the incidence of shortened inter-insemination intervals. Heifers (n = 47) assigned to the TAI group which came into estrus between the time of $PGF_{2\alpha}$ injection and the second administration of GnRH-agonist were inseminated on Day 8 or Day 9 at 0800 h according to the time of estrus occurrence . This procedure was followed since heifers detected in estrus at this early stage would not likely conceive to the timed-insemination made 63 h after injection of $PGF_{2\alpha}$. This decision would minimize economic losses to the cooperating commercial farm. These heifers did not receive the second Buserelin injection. The remaining heifers in the TAI group (n =140) were inseminated at 15 h after the second injection of GnRH-agonist (Day 10, 0800 h). Heifers in the IDE group were inseminated 0 to 12 h after observation of estrus behavior as described for the first field study. In the first replicate of this experiment, blood was collected from all heifers of both groups at the time of the second GnRH-agonist injection and again 10 d later (mid-luteal phase) for measurement of plasma progesterone concentrations. Blood was collected into heparinized tubes (Vacutainer, Becton Dickinson Vacutainer Systems USA, Rutherford NJ) by jugular venipuncture and stored in an ice bath until plasma was separated by centrifugation (1,800 g for 30 min) The separated plasma was kept on ice for 10 h during transport and stored at -20° C until assayed for progesterone (Knickerbocker et al., 1986). Intra- and inter-assay coefficients of variation were 8.5 and 12 %. The purpose of this sampling was to: 1) assess CL status 48 h after administration of $PGF_{2\alpha}$ and 2) assess luteal function at approximately 9 d after ovulation of a follicle with a GnRH-agonist.

Experiment 3: hCG at 48 h After $PGF_{2\alpha}$

The third field study was designed to test the efficacy of using a timed-insemination following injection of hCG 48 h after administration of $PGF_{2\alpha}$ (Figure 7-3). Heifers (TAI, n=102; IDE, n=98) were managed as in Experiment 2, except that the second injection of GnRH-agonist for the TAI group, was replaced by an

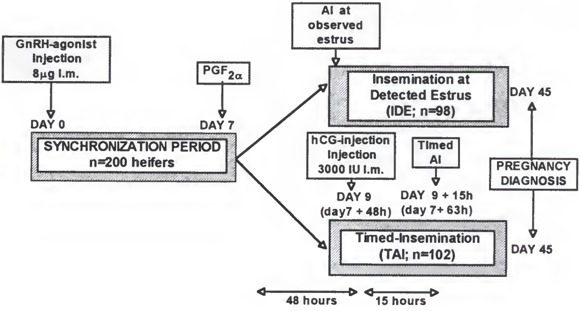

GnRH-agonist Injection 8µg i.m.
PGF2α
DAY 0
DAY 7
SYNCHRONIZATION PERIOD n=200 heifers
AI at observed estrus
Insemination at Detected Estrus (IDE; n=98)
DAY 45
hCG-injection Injection 3000 IU i.m.
Timed AI
PREGNANCY DIAGNOSIS
DAY 9 (day7 + 48h)
DAY 9 + 15h (day 7+ 63h)
Timed-Insemination (TAI; n=102)
DAY 45
48 hours
15 hours

Figure 7-3: Experimental design for the third timed-insemination experiment.

administration of hCG (3000 IU i.m.; Steris Laboratories, Phoenix AZ) 48 h after the injection of $PGF_{2\alpha}$ (Day 9 at 1700 h). It was hypothesized that the period of LH-like stimulation of the ovulatory follicle due to the extended persistence of hCG in the blood (Seguin et al., 1977, Chapter 4) should stimulate the ovulatory follicle and improve its differentiation into a CL with increased steroidogenicity and lifespan and help to further reduce the incidence of shortened inter-insemination intervals observed in the second experiment. Heifers (n = 23) assigned to the TAI group which came into estrus between the time of $PGF_{2\alpha}$ injection and the administration of hCG were inseminated on Day 8 or Day 9 at 0800 h according to the time of estrus occurrence as done for Experiment 2. These heifers did not receive the injection of hCG. The remaining heifers in the TAI group (n =79) were inseminated at 15 h after the injection of hCG (Day 10, 0800 h). Heifers in the IDE group were inseminated 0 to 12 h after observation of estrus behavior as described for the previous experiments.

Statistical Analyses

Data were analyzed using the general linear model procedures (GLM) and the categorical data procedures (CATMOD) of the Statistical Analysis System (SAS, 1988). Heifers were considered to be responding to the synchronization protocol when they showed estrus signs within a 7 d period after administration of $PGF_{2\alpha}$. Pregnancy rate was the proportion of the heifers pregnant which were assigned to a treatment. Conception rate was the proportion of the heifers pregnant which were inseminated at observed estrus. In the TAI group, all heifers were inseminated (before or after the second GnRH-agonist injection); thus conception rates were the same as pregnancy rates. The inter-insemination interval was the time between two consecutive inseminations. Inter-insemination intervals were classified as short if they were less than or equal to 16 d and normal if they were greater than 16 d or if the synchronized heifers were pregnant. For the first and third experiments, pregnancy and conception rates as well as inter-insemination intervals were analyzed using models which included the effects of treatment, bull and the interaction of treatment x bull. For the second experiment, conception rates, pregnancy rates and inter-insemination intervals for the two replicates were analyzed with models including the effects of treatment, bull, replicate, and the interactions treatment x bull, treatment x replicate, bull x replicate and treatment x bull x replicate. The effect of treatment was tested against the treatment x bull interaction; the effect of replicate was tested against the replicate x bull interaction; the replicate x treatment interaction was tested against the replicate x treatment x bull interaction.

Results

Experiment 1: GnRH at 24 h After $PGF_{2\alpha}$

Synchronization, pregnancy and conception rates. Control heifers, that were inseminated at detected estrus had a very high synchronization rate (85%; 68/80) with most of the estrous activity (94.1%; 64/68) occurring between 24 and 96 h after $PGF_{2\alpha}$ injection (Table 7-1).

Table 7-1. Numbers and percentages of heifers detected in estrus during the 7 d following $PGF_{2\alpha}$ injection for the heifers inseminated at detected estrus (IDE) for the first, second and third experiments (replicates 1 and 2).

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
First Experiment: Number (percent) of heifers in estrus	0	8 (11.7%)	42 (61.8%)	14 (20.6%)	3 (4.4%)	0	1 (1.5%)
Second Experiment: Number (percent) of heifers in estrus	18 (13%)	20 (14.4%)	63 (45.3%)	21 (15.1%)	6 (4.3%)	8 (5.8%)	3 (2.1%)
Third Experiment: Number (percent) of heifers in estrus	6 (7.9%)	5 (6.6%)	3 (69.7%)	6 (7.9%)	2 (2.6%)	4 (5.3%)	0

Pregnancy rates in TAI were reduced compared with IDE (25.8% < 48.7%; $P < .0005$). Likewise conception rates also were reduced for the TAI group when compared to the heifers in the IDE group (25.8% < 57.4%; $P < .0001$; Table 7-2.)

Table 7-2. Estrus, conception and pregnancy rates for Experiment 1

Response	Insemination at Detected Estrus	Timed-Insemination
Heifers in study	80	89
Estrus detection rate Heifers in estrus during the 7 d after $PGF_{2\alpha}$	85% (68/80)	
Pregnancy rates [a]	48.7% (39/80)	25.8% (23/89)
Conception rates [b]	57.4% (39/68)	25.8% (23/89)

[a] Insemination at detected estrus > timed-insemination; *P*<.0005
[b] Insemination at detected estrus > timed-insemination; *P*<.0001

Inter-insemination interval. A greater incidence of short inter-insemination intervals ($\leq$ 16 d) was detected for the TAI group compared to heifers inseminated at detected estrus (34.8% > 2.9%; *P* < .001; Table 7-3).

Table7-3. Frequency distribution of the inter-insemination intervals for the heifers that did not conceive according to treatment (Experiment 1).

	Insemination at detected estrus (n=68)	Timed-insemination (n=89)
Short inter-insemination interval $\leq$16 d[a]	2.9% (2/68)	34.8% (31/89)

[a] Insemination at detected estrus < timed-insemination; *P*<.001

Experiment 2: GnRH at 48 h After $PGF_{2\alpha}$

Synchronization, pregnancy and conception rates. The second experiment was replicated. Pregnancy rates were not affected by the effect of Replicate or Bull (Table 7-4).

Table 7-4. Least squares analysis of variance for the effect of timed-insemination on pregnancy rates

Source	d.f.	Mean Square	Error term
Replicate[a]	1	.000057 NS[a]	Replicate x Bull
Treatment[b]	1	.001851 NS	Treatment x Bull
Replicate x Treatment[c]	1	.272389 NS	Replicate x Treatment x Bull
Bull[d]	4	.300361 NS	Residual
Replicate x Bull	4	.143475 NS	Residual
Treatment x Bull	4	.065255 NS	Residual
Replicate x Treatment x Bull	4	.193728 NS	Residual
Residual	344	.255456	

[a] Non Significant (NS)

Results of the two replicates were pooled for presentation (Table 7-5). Heifers in the IDE group had a high synchronization rate (78.5%; 139/177) with most of the estrous activity (88.8%; 122/139) occurring between 0 and 96 h after $PGF_{2\alpha}$ injection (Table 7-1). Pregnancy rates (Table 7-5) were not different for the two treatments (48.0%, IDE vs 45.5%, TAI).

Table 7-5. Estrus, pregnancy and conception rates for Experiment 2

Response	Insemination at detected estrus	Timed-insemination
Heifers in study	177	187
Estrus detection rate (heifers in estrus during the 7 d after $PGF_{2\alpha}$)	78.5% (139/177)	
Pregnancy rates [a]	48.0% (85/177)	45.5% (85/187)
Conception rates [b]	61.2% (85/139) [e]	45.5% (85/187)
Conception rates 15 or 39 hours after $PGF_{2\alpha}$ [c]	55.3% (21/38)	61.7% (29/47) [e]
Conception rates day 2 to 7 after $PGF_{2\alpha}$ (IDE) 63 h after $PGF_{2\alpha}$ (TAI) [d]	63.4% (64/101)	40% (56/140) [e]

[a] NS

[b] Insemination at detected estrus > timed-insemination; *P*<.005

[c] NS

[d] Insemination at detected estrus > timed-insemination; *P*<.001

[e] Conception rates 63 h after $PGF_{2\alpha}$ < conception rates 15 or 39 h after $PGF_{2\alpha}$ for heifers in TAI; $P < .01$

For experiment 2, conception rates for each insemination system are reported separately for heifers 1) bred to a standing estrus within 39 h after injection of $PGF_{2\alpha}$ and 2) bred between 2 and 7 d after injection of $PGF_{2\alpha}$ (Table 7-5). This distinction was made because of heifers (n=47 of 187) assigned to the TAI treatment which came into estrus before their second injection of GnRH-agonist (in Experiment 1, this did not occur since the second injection of Buserelin was given

24 h after administration of $PGF_{2\alpha}$). Heifers in this early time period conceived at a similar rate regardless of insemination system (61.7 vs 55.3%). This was not surprising because all animals had followed the same protocol up to this point. During the second period of insemination (d 2 to 7 after $PGF_{2\alpha}$ injection), heifers assigned to the TAI group conceived at a lower rate ($P < .001$) than heifers bred to a standing estrus (40 vs 63.4%). This resulted in a lower overall conception rate for heifers assigned to the TAI group (41.5 vs 61.2%; $P < .005$). In the TAI group however, all heifers were presented for insemination in a 3-d period *versus* a 40-d period for heifers in the control group.

Inter-insemination interval. There was a greater occurrence of short inter-insemination intervals ($\leq$ 16 d) in the TAI group (15.5%) than the IDE group (4.3%). No difference was detected in the occurrence of short inter-insemination intervals in heifers that were inseminated during the 39 h after injection of $PGF_{2\alpha}$ (TAI, 0% vs IDE, 2.6%; $P<.44$; Table 7-6). However, the occurrence of short inter-insemination intervals was increased in heifers in TAI compared with IDE heifers (15.5% > 4.3% ; $P<.015$), when all heifers inseminated during the 7 d after $PGF_{2\alpha}$ are considered (Table 7-6). The occurrence of short inter-insemination intervals differed between experiment 1 and 2 (TAI of first Experiment [34.8%] > TAI of second Experiment [15.5%]; $P<.01$). Delaying the second injection of GnRH-agonist until 48 h after injection of $PGF_{2\alpha}$ reduced occurrence of short inter-insemination intervals in the timed-insemination group.

Table 7-6. Frequency distribution of the inter-insemination intervals in heifers that did not conceive according to treatment (Experiment 2; replicates 1 and 2).

Response of all experimental heifers	Insemination at detected estrus (n=139)	Timed-insemination (n=187)
Short inter-insemination interval ($\leq$16 d) [a]	4.3% (6/139)	15.5% (29/187)
Response of experimental heifers inseminated at 15 or 39 hours after $PGF_{2\alpha}$	**Insemination at detected estrus (n=38)**	**Timed-insemination (n=47)**
Short inter-insemination interval ($\leq$16 d) [b]	2.6% (1/38)	0% (0/47)

[a] Insemination at detected estrus < timed-insemination; $P<.001$
[b] N.S.

Plasma progesterone. Forty-eight hours after injection of $PGF_{2\alpha}$, 79.6% of the heifers had a concentration of plasma progesterone $\leq$ 2 ng/ml, indicative of a regressed or regressing CL. The distribution of heifers experiencing a short inter-insemination interval did not differ in frequency when categorized by luteal status (less or greater than 2 ng/ml P4) at 48 h after injection of $PGF_{2\alpha}$ (Table 7-7).

Table 7-7. Frequency distribution of the inter-insemination intervals according to the plasma concentrations of progesterone 48 h after injection of $PGF_{2\alpha}$ for the heifers of the TAI group inseminated 15 h after the second injection of GnRH-agonist during the first replicate (Experiment 2)

Progesterone at 48 h after $PGF_{2\alpha}$	Plasma progesterone $\leq$ 2 ng/ml (n=50)	Plasma progesterone > 2 ng/ml (n=18)
Synchronized heifers with inter-insemination interval $\leq$ 16 d	14.7% (10/68)	8.8% (6/68)

The cumulated frequency of pregnancies regressed against the concentration of progesterone at 48 h after the second injection of $PGF_{2\alpha}$ was not different between TAI and IDE. Heifers having short estrous cycles had lower plasma concentrations of progesterone at 12 d after $PGF_{2\alpha}$ than heifers not observed in estrus after insemination (pregnant heifers or missed estrus) or with inter-insemination intervals longer than 16 d ($3.9 \pm .9$ ng/ml < $10.3 \pm .3$ ng/ml; $P<.001$). A high proportion of heifers (50%) having short inter-insemination intervals had low (≤ 2 ng/ml) plasma concentrations of progesterone 12 d after $PGF_{2\alpha}$.

Experiment 3: hCG at 48 h After $PGF_{2\alpha}$

Synchronization, pregnancy and conception rates. Heifers in the IDE group had a high synchronization rate (77.5%; 76/98; Table 7-1). Pregnancy rates (Table 7-8) were not different for the two treatments (56.1%, IDE vs 52.9%, TAI). As in experiment 2, conception rates for each insemination system are reported separately for heifers 1) bred to a standing estrus within 39 h after injection of $PGF_{2\alpha}$ and 2) bred between 2 and 7 d after injection of $PGF_{2\alpha}$ (Table 7-8). Heifers in this early time period conceived at a similar rate regardless of insemination system (63.6%, IDE *vs* 56.5%, TAI). During the second period of insemination (d 2 to 7 after $PGF_{2\alpha}$ injection), heifers assigned to the TAI group conceived at a lower rate ($P < .01$) than heifers bred to a standing estrus (51.9%

vs 73.9%). This resulted in a lower overall conception rate for heifers assigned to the TAI group (52.9 vs 72.3%; $P < .005$). In the TAI group however, all heifers were presented for insemination in a 3-d period *versus* a 40-d period for heifers in the control group as reported for Experiment 2.

Table 7-8. Estrus, pregnancy and conception rates for Experiment 3

Response	Insemination at detected estrus	Timed-insemination
Heifers in study	98	102
Estrus detection rate (heifers in estrus during the 7 d after $PGF_{2\alpha}$)	77.5% (76/98)	
Pregnancy rates [a]	56.1% (55/98)	52.9% (54/102)
Conception rates [b]	72.3% (55/76)	52.9% (54/102)
Conception rates 15 or 39 h after $PGF_{2\alpha}$ [c]	63.6% (7/11)	56.5% (13/23) [e]
Conception rates day 2 to 7 after $PGF_{2\alpha}$ (IDE) 63 h after $PGF_{2\alpha}$ (TAI) [d]	73.9% (48/65)	51.9% (41/79) [e]

[a] NS

[b] Insemination at detected estrus > timed-insemination; $P < .01$

[c] NS

[d] Insemination at detected estrus > timed-insemination; $P < .01$

[e] NS; Conception rates 63 h after $PGF_{2\alpha}$ did not differ from conception rates 15 or 39 h after $PGF_{2\alpha}$ for heifers in TAI.

Inter-insemination interval. There was no differences in the occurrence of short inter-insemination intervals (Table 7-9) in the TAI group (6%) or in the IDE group (1%).

Replacing the second injection of GnRH-agonist 48 h after administration of $PGF_{2\alpha}$ by an injection of hCG, reduced the occurrence of short inter-insemination intervals in the timed-insemination group to levels similar to that of heifers inseminated at detected estrus.

Table 7-9. Frequency distribution of the inter-insemination intervals in heifers that did not conceive according to treatment (Experiment 3).

	Insemination at detected estrus (n=96)	Timed-insemination (n=102)
Short inter-insemination interval $\leq$16 d [a]	1% (1/96)	6% (6/102)

[a] N.S.

[b] N.S.

Discussion

Wolfenson et al. (1994) reported that lactating cows, synchronized with a GnRH-agonist injection on d 12 of the cycle followed 7 d later by an injection of $PGF_{2\alpha}$, had more precise synchronization of estrus than cows treated with a single injection of $PGF_{2\alpha}$ on d 12 of the estrous cycle. The GnRH-agonist injection synchronized follicular development and resulted in a more homogeneous wave of follicular development (Thatcher et al., 1989; Macmillan and Thatcher, 1991; Twagiramungu et al., 1994; Wolfenson et al., 1994). The coordination of follicular growth and luteal regression likely are responsible for improved synchronization of estrus (Fogwell et al., 1986). Similar results were observed in the present experiments since 75% (267/355) of the heifers in the control groups were detected in estrus from d 0 to d 4 after injection of $PGF_{2\alpha}$ for the three experiments.

It is likely that the reduced pregnancy and conception rates of heifers in the TAI group of the first experiment were due largely to occurrence of short estrous cycles. The difference in short inter-insemination intervals between Experiment 1 and 2 (34.8% vs 15.5%) was of the same order of magnitude as the difference in conception rates between the first and second experiments (31.6% vs 15.7%).

The occurrence of short inter-estrous intervals was not reported by others (Pursley et al.,1994a; Twagiramungu et al., 1995) working with multiparous cows. Since short inter-insemination intervals occured only in the heifers that received the second injection of Buserelin (TAI group), it appears that the first part of the synchronization protocol (GnRH-agonist followed 7 d later by $PGF_{2\alpha}$) did not directly cause the short cycles. Therefore short inter-insemination intervals are unlikely the result of the small proportion of heifers forming an accessory CL after treatment with the first injection of GnRH-agonist when compared to cows (Pursley et al., 1994b).

The large occurrence of short inter-insemination intervals in heifers may be due to differences in the timing of spontaneous ovulation between cows and heifers after synchronization with injections of GnRH-agonist followed 7 d later by $PGF_{2\alpha}$ (Silcox et al.; 1995). Lactating dairy cows develop larger follicles that are less estrogenic earlier during the preovulatory period, when compared to non-lactating cows (De la Sota et al., 1993). This may cause the preovulatory follicles to respond differently to a surge of LH induced by administration of a GnRH-agonist. Perhaps the delayed growth of preovulatory follicles in non-lactating cows could explain the higher frequency of short estrus cycles observed in heifers treated with the timed-insemination protocol. For example, the second injection of the GnRH-agonist would induce a surge of LH too early in the development of the preovulatory follicle. This preovulatory follicle, due to an inadequate maturation, would be unable to ovulate or would produce a corpus luteum with a reduced lifespan. Delaying the second GnRH-agonist injection until 48 h after injection of $PGF_{2\alpha}$ in the second experiment improved pregnancy rates and reduced the occurrence of short inter-insemination intervals (34.8 > 15.5%; *P*<.001) when compared with experiment 1.

Alternatively occurrence of short estrous cycles and poor conception and pregnancy rates of the first experiment may be due to failure of CL regression or incomplete maturation of the follicle and/or oocyte at the time of ovulation. Regression of the CL after administration of $PGF_{2\alpha}$ could be impaired by the second injection of the GnRH-agonist given this early. This would lead to delayed regression of the CL and estrus occurring before d 17 after insemination for heifers in the TAI group. Donaldson and Hansen (1965) suggested that stimulation with LH in the late luteal phase may extend CL function. However, occurrence of short inter-insemination intervals in the second experiment (GnRH-agonist injection delayed until 48 h) was not related to the plasma concentration of progesterone at the time of the second injection of GnRH-agonist for the heifers in the TAI group

(Table 7-7). Only 26% of the heifers that were timed inseminated had a plasma concentration of progesterone greater than 2 ng/ml. Of these 26% (n=18), 12 heifers (67%) became pregnant or had estrous cycles of normal length (> 16 d). Thus failure of luteal regression is not the primary cause of the increased frequency of short inter-insemination intervals in heifers of the TAI group receiving the second administration of GnRH-agonist at 48 h. This is in agreement with observation of Litch and Condon (1988) that reported no effect of hCG administered before and at the time of injection of $PGF_{2\alpha}$ on the *in vitro* progesterone production of bovine CL. Seguin et al. (1977) also observed that hCG administered in the proestrus period (d 17), after the initial decline in progesterone due to CL regression, did not extend estrous cycle in heifers.

Furthermore, when the second injection of Buserelin was replaced by an injection of hCG, in the third experiment, the occurrence of short inter-insemination intervals was reduced to levels comparable to those observed for heifers of the IDE group. Therefore the occurrence of short estrus intervals in the first and second experiment is likely due to an inadequate gonadotropic stimulation of the ovulatory follicle.

Ovulation of a first wave dominant follicle can be induced with an injection of GnRH-agonist (Buserelin, 8 µg) or 3,000 IU of hCG (Schmitt et al., 1993; Schmitt et al., 1994a). However, the CL resulting from the GnRH-agonist induced ovulation had a reduced capability to secrete progesterone compared to the CL induced by hCG (Schmitt et al., 1994a). It must be noted that a single injection of Buserelin (10 µg, i.m.) elicited an LH release that lasted 5 h in heifers (Chenault, 1990), which is approximately half the length of a naturally occurring (Chenault et al, 1975; Rahe et al., 1980) or a $PGF_{2\alpha}$ induced (Chenault et al., 1976) pre-ovulatory surge of LH in cattle. The normal 10-h LH surge may induce a better stimulation of the ovulatory follicle and formation of a subsequent CL with a normal lifespan. In cattle, hCG persists in plasma for an extended period of time following an i.m. injection (Seguin et al., 1977). When hCG was administered (i.m.) at a dose of 3000 IU, hCG was detected in plasma of heifers until 66 h after injection (Chapter 4). In primates, the normal LH surge lasts 48 to 50 h (Hoff et al., 1983). Injections of GnRH or GnRH-agonist induce a transient LH surge of 10 to 24 h that is insufficient to elicit maturation of the oocyte or support early luteal function in primates (Zelinski-Wooten et al., 1991). An injection of hCG in primates maintain LH-like bioactivity for 72 h after injection and induced normal follicular and oocyte maturation followed by formation of a normal steroidogenic CL (Chandraseckher et al., 1991, Zelinski-Wooten et al., 1991). It was hypothesized that a longer stimulation of the follicle by hCG (50 to 72 h) induced a more complete luteinization of granulosa cells (Chandraseckher et al., 1991). In addition, Jones (1991) reported that a single LH elevation induced by an injection of GnRH in primates was not sufficient to occupy all the receptors of granulosa cells nor induce the formation of a CL with a normal lifespan.

In cattle, the increase in pulsatile frequency of LH and baseline concentrations observed between CL regression and the pre-ovulatory surge of LH

(Rahe et al., 1980) may enhance maturation of the ovulatory follicle. The lower incidence of short estrous cycles observed in the second experiment suggested that delaying the second injection of GnRH-agonist by 24 h (48 h after $PGF_{2\alpha}$) allowed more time between CL regression and the ovulatory surge of LH for development of the ovulatory follicle. Other authors (Lucy et al., 1986a,b) reported reductions in plasma progesterone concentrations during the luteal phase following ovulation induced with an injection of GnRH-agonist 72 h after the second injection of $PGF_{2\alpha}$ (two treatments with $PGF_{2\alpha}$ given 11 d apart) and 8 h before a timed-insemination. These results support the possibility that the GnRH-agonist injection may produce an incompetent CL in a proportion of heifers whose follicles were not sufficiently mature at the time the GnRH-agonist was injected. In addition, when hCG was administered 48 h after injection of $PGF_{2\alpha}$ in Experiment 3, the occurrence of short inter-insemination intervals in the TAI group was similar to what is observed in the control group.

In this last experiment, pregnancy rates did not differ between the two treatment groups. However, conception rates were lower for heifers in the TAI group. Perhaps the reduction in conception rates is due to the heifers that did not respond to the synchronization protocol (first injection of GnRH-agonist followed 7 d later by an injection of $PGF_{2\alpha}$) but are included in the calculation of the conception rates in the TAI group. This hypothesis is supported by the absence of differences in conception rates for heifers in the TAI group that are inseminated at observed estrus (15 or 39 h after $PGF_{2\alpha}$; 56.5%) or that are timed-inseminated (63 h after $PGF_{2\alpha}$; 51.9%).

The reduction in conception rates detected in the three experiments also could be due to a failure of ovulation. However, recent reports (Pursley et al., 1994b; Schmitt et al., 1994b; Silcox et al., 1995) showed that the second injection of GnRH-agonist caused ovulation of the dominant follicle recruited by the first injection of GnRH-agonist. These authors confirmed that ovulation occured 24 to 32 h after the second GnRH-agonist injection (given 24 or 48 h after $PGF_{2\alpha}$ in heifers and cows).

It also must be noted that the period of increased pulsatile LH frequency between luteolysis and the LH surge (Rahe et al., 1980) is essential for resumption of the ribosomal RNA transcription in cattle oocytes (Greve et al., 1995). This is characterized at the cellular level by vacuolization of the oocyte nucleolus. If the surge of LH induced by administration of the GnRH-agonist occurs before ribosomal RNA transcription takes place, further resumption of meiosis may be impaired. The subsequent oocyte could be incompetent and may have contributed to the reduced fertility of the TAI group. Delaying the ovulatory injection of GnRH-agonist to 48 h after injection of $PGF_{2\alpha}$ may allow time for resumption of ribosomal RNA transcription in cattle oocytes. In Experiments 2 or 3, a delay of the ovulatory injection of Buserelin or hCG to 48 h after $PGF_{2\alpha}$ reduced the incidence of short estrous cycles and improved pregnancy rates. A greater exposure to LH-like activity via injection of hCG eliminated the higher frequency of shortened cycles perhaps due to a greater luteinization and differentiation of the ovulatory follicle compared to that induced by a GnRH-agonist.

Further experimentation needs to determine whether this advantage of hCG in heifers can be utilized in reducing the interval to hCG injection to 36 h after $PGF_{2\alpha}$. This would permit a timed-insemination to all experimental heifers without the need of inseminating early detected estrus (< 39 h after administration of $PGF_{2\alpha}$). Additional research is warranted to develop a GnRH or GnRH-agonist releasing system that would induce a more prolonged release of LH and eliminate the need for hCG which is immunogenic in cattle.

Implications

This ovulatory control system allowed 100% of the heifers to be presented for AI in 3 d versus 40 d for heifers of the control group (Experiments 2 and 3) without reduction in pregnancy rates. Replacing the second injection of GnRH-agonist with hCG eliminated the occurrence of short estrous cycles. Implementing such a protocol for TAI could be useful in situations where estrus detection is not possible or difficult (post partum dairy cows with reduced estrous behavior under heat stress conditions). Perhaps hCG injected 36 h after administration of $PGF_{2\alpha}$ may completely eliminate the need for estrus detection without reduction of pregnancy rates.

CHAPTER 8
GENERAL DISCUSSION AND CONCLUSIONS

Pregnancy rate in cattle is the product of heat detection and conception rate. In current management practices of the dairy herd, there is a strong economical advantage to manage reproduction and synchronize estrus (Macmillan, 1992). This is emphasized in situations where heat detection is made difficult (i.e., heat stress conditions, post-partum period). Objectives of this dissertation were to evaluate the use of a GnRH-agonist, hCG and $PGF_{2\alpha}$ to regulate follicle and corpus luteum function in cattle.

Five series of experiments were designed to elucidate the effect of these pharmaceutical agents on follicular dynamics and define new strategies for estrus synchronization and improvement of reproductive efficiency in cattle. In chapter 3, it was determined if an injection of a GnRH-agonist would eliminate a persistent first wave dominant follicle with reduced fertility and induce recruitment of a new dominant follicle with improved fertility. In Chapters 4, 5, 6, the gonadotropic requirements of a first wave dominant follicle for ovulation and formation of a functional CL were examined. The first wave dominant follicle was our model for induction of ovulation with hCG or a GnRH-agonist. The effect of an administration of hCG on day 5 of the estrous cycle on conception rates in lactating dairy cows, exposed to the heat stress conditions of summer and in fertile Holstein heifers was tested. Finally, in Chapter 7, knowledge gleaned from Chapters 3 to 6 was used to design a protocol to synchronize ovulation and allow timed-insemination. This would eliminate the need for estrus detection.

On the basis of many reports (Sirois and Fortune, 1990; Savio et al., 1993b; Custer et al., 1994), norgestomet implants and other progesterone releasing devices used for estrus synchronization in cattle allow a certain percentage of animals to develop persistent follicles when treatment is initiated during the second part of the estrous cycle.

Persistent follicles induce an estrus with reduced fertility upon ovulation when compared to ovulation of a newly recruited dominant follicle (Chapter 3). This reduced fertility was attributed to early embryonal mortality or failure of fertilization (Mihm et al., 1994). Oocytes collected from persistent dominant follicles underwent premature resumption of meiosis and had started germinal vesicle breakdown while still in the follicle. Low fertility after ovulation of follicles with prolonged periods of dominance could be due to asynchronous nuclear and cytoplasmic maturation of the oocyte. Ahmad et al. (1995) reported that embryonic losses in animals with persistent follicles occurred during the early cleavage stages, when zygotes are still in the oviduct. They suggested that increased plasma estradiol altered oocyte maturation or oviductal function. Although oocytes collected from persistent follicles appear to undergo premature development, they underwent normal embryo development if matured, fertilized and cultured in vitro (Borchert et al., 1995). This further supports an alteration of the in vivo oviductal and / or uterine environment. Binelli and Thatcher (Unpublished observations) demonstrated an alteration in protein synthesis by oviductal tissue collected from cows at estrus that had a persistent follicle.

On the basis of these findings, treatments designed for induction of estrus and ovulation with progestogens must either use sufficiently high doses of progestins to induce regular turnover of the dominant follicle, or include some other treatment that will assure follicular turnover if high fertility is to be achieved. Such a treatment could be an additional administration of progesterone (Anderson and Day, 1994), an injection of estrogens early during the progestin treatment (Bo et al., 1994), or treatment with a GnRH agonist 7 to 9 days before removal of the progestin implant (Chapter 3). At the present time, GnRH or GnRH-agonists are commercially available and could be used in synchronization programs with the use of progestins. It is possible that a GnRH-agonist, since they induce a concomitant surge of LH and FSH (Chenault et al., 1990), may induce recruitment of a new ovulatory follicle faster and better synchronized than estradiol or progesterone. The different GnRH-agonists available have different potency (Chenault et al., 1990; Thatcher et al., 1993). Buserelin, with its greater potency (80 times more potent than native GnRH as measured by LH and FSH release) may have more repeatable effects on a population basis than other GnRH-agonist or native GnRH

Estrogens should be administered in a short acting preparation (estradiol-benzoate) to avoid any long term effects of estrogens released in a slow release matrix (e.g. estradiol-valerate). Estradiol valerate is the long-acting estrogen utilized with the Syncro-Mate B protocol (Bo et al., 1995; Larson and Kiracofe, 1995) and induces estrous behavior in the absence of the ovary or follicular development.

If progestins are used to induce dominant follicle turnover, one must keep in mind that the different progestins do not have the same regulatory effect on LH secretory patterns. For instance, MGA used at 3 times the dose that is normally used for estrus synchronization did not mimic endogenous progesterone in regulation of LH secretion, but induced a secretory pattern characteristic of the follicular phase (Kojima et al., 1995). This is important to avoid formation of a persistent dominant follicle.

Ovulation can be induced with different hormones in cattle. However, three factors need to be considered: LH priming of the follicle prior to LH surge, duration of the surge of LH and LH support of the follicle after the pre-ovulatory surge. The first wave dominant follicle can be induced to ovulate with either a GnRH-agonist or hCG injection. The first wave dominant follicle on day 5 of the estrous cycle is healthy, estrogen active and will form a functional corpus luteum after induction of ovulation with either hCG or GnRH (Chapter 4). However, the increase in progesterone is much greater after hCG treatment. This is due to induction of a larger and more functional corpus luteum with a minimal effect of hCG or GnRH-agonist on the original corpus luteum (Chapter 5, Experiment 1 and Experiment 2).

This differential effect of hCG was attributed to the longer half-life of this molecule but could not be explained after microscopic studies of the induced or original CL. Due to its longer half-life (Chapter 4), hCG may possibly stimulate a greater mitotic activity of theca cells and then small luteal cells during the peri-ovulatory period. Since theca cells were shown to divide during the early stages of CL formation (Donaldson et al., 1965b, Zheng et al., 1994), an increased mitotic activity may explain the formation of a larger CL.

Detailed study of the ultrastructure of luteal cells of the induced and original CL, after treatment with a GnRH agonist or hCG on day 5 of the estrous cycle (Chapter 5), revealed that luteal cells progress through three stages of development leading up to a final stage of regression. Treatment with hCG or GnRH-agonist delays the progression through those stages of development. These observations suggest that the additional LH delays the progression of small and large luteal cells through, stages of development leading to CL regression. Perhaps extension of the CL lifespan, when cows are treated with LH during the second part of the estrous cycle (Donaldson and Hansel, 1965b), is due to the increased number of Stage 1 and Stage 2 cells that are less sensitive to the luteolytic effect of $PGF_{2\alpha}$ (Fields et al., 1991b).

Furthermore, the increased progesterone synthesizing capacity of the hCG-induced CL (Chapter 5, Experiment 1) may be due to healthier Stage 1 and 2 cells. In this case hCG, due to its longer half-life, would maintain the luteal cells in Stage

1 and retard the process of degeneration for a longer period of time than the GnRH-agonist.

However, no inference can be made from the experimental approach taken regarding transformation of small luteal cells into large luteal cells (Donaldson and Hansel, 1965; Alila and Hansel, 1984; Meidan et al., 1990). Finally, ultrastructural evidence could explain the increased steroidogenic capacity of the hCG induced CL when compared to the GnRH induced CL. In Chapter 5, there is a slightly higher percentage of Stage 1 small and large luteal cells in the hCG induced CL when compared to the GnRH induced CL ($P < .1$). They may indicate a trend for hCG, perhaps because of its longer half-life, to retard progression of the luteal cells through the stages of development when compared to GnRH-agonist. However, Litch and Condon (1988) did not report any effect of hCG administered before and at the time of $PGF_{2\alpha}$ injection on in vitro progesterone production of bovine CL. The higher content of progesterone in unincubated tissue of hCG and GnRH-agonist original (d 17) CL (Chapter 5) indicated that luteal cell involution was indeed retarded by the gonadotropin treatment. Diaz et al. (1993) demonstrated that cattle treated with hCG on d 5, all had three wave follicle cycles and a slightly longer estrous cycle. Therefore, a delay in luteolytic signaling (e.g. estradiol and $PGF_{2\alpha}$) may have occurred and this is reflected by the lower frequency of Stage 3 luteal cells in hCG and GnRH-agonist treated cows. Further experimentation is warranted to study the influence of LH stimulation on the process of luteal cells passing through the different morphological stages leading up to regression.

Establishment and maintenance of pregnancy as well as embryo growth in cattle is related to the ability of the CL to secrete progesterone (Staples and Hansel, 1961; Estergreen and al., 1968). Several reports suggest that a deficiency in the steroidogenic secretion of the CL, leading to low concentrations of progesterone, could be a factor contributing to embryo losses (Staples and Hansel, 1961, reviewed by Thatcher et al., 1994).

A GnRH-agonist or hCG administration on d 5 of the estrous cycle consistently induces formation of an additional CL (Chapter 4, Chapter 5) and is a strategy to increase concentrations of progesterone in plasma during the luteal phase. However, such a treatment did not increase conception rates in lactating dairy cows during the heat stress conditions of summer or in fertile Holstein heifers (Chapter 4). Heat stress induces damage of the oocyte on the day of estrus (Putney et al., 1988a) and the embryo early after fertilization (Ealy et al., 1993). The increase of progesterone induced by hCG treatment probably occurred too late to be of any beneficial effect. Furthermore in heifers, fertility is much higher with late stages embryonic losses estimated to be only 10% (Thatcher et al., 1994) and the progesterone secretion during the luteal phase is not altered by the metabolic demands of lactation.

Additional research is warranted to test this hCG treatment protocol in lactating dairy cows not submitted to the heat stress conditions. In this case the higher metabolic demand of the postpartum lactation may induce low concentrations of progesterone in plasma and embryonic mortality may be due to earlier corpus luteum regression at a time when the embryo is signaling presence to the maternal unit.

Corpora lutea are a continuation of follicular maturation (McNatty, 1979) and the preparation of luteal cells for synthesis and secretion of progesterone begins before ovulation. In fact ovulation is not a prerequisite for luteinization and secretion of progesterone in cattle (Kesler et al., 1981). Subnormal luteal function may result from inadequate stimulation of the preovulatory follicle by gonadotropins.

In cattle, preovulatory follicular maturation results from the coordinated actions of FSH and LH on granulosa and theca cells, respectively. The theca interna synthesizes androgens in response to LH stimulation. Androgens enter the granulosa cells where they are converted to estradiol-17β by aromatase, an enzyme regulated by FSH (Richards, 1980; Hansel and Convey, 1983, Fortune and Quirk, 1988). Follicule stimulating hormone is associated with antrum formation, growth of the antral follicle, granulosa cell viability, and synthesis of gonadotropin receptors on granulosa cells is under control of FSH (McNatty, 1979, Richards, 1980; Richards and Heidin, 1988). Moreover, when a follicle does not receive sufficient LH support, production of androgens is limited. As a consequence, synthesis of estradiol and therefore formation of LH receptors on granulosa cells as well as the mitosis of granulosa cells is impaired (Richards, 1980; Fortune and Quirk, 1988).

These phenomena may be of importance when a follicle that did not receive adequate LH support was induced to ovulate prematurely (Chapter 6). The subsequent corpus luteum did not secrete adequate levels of progesterone. Perhaps a low level of LH receptors on granulosa cells of the follicle did not allow adequate luteinization of the granulosa cells and subsequent differentiation into functional luteal cells.

Furthermore, since number of granulosa cells is reduced in the absence of mitotic stimulation of estradiol (Richards, 1980), the subsequent corpus luteum may contain less large luteal cells and therefore be less steroidogenic (O'Shea, 1987).

Finally, increased estradiol by the proestrous follicle may enhance the pre-ovulatory surge of LH (Chapter 6). Increased estradiol secretion may be acting on the hypothalamus to increase secretion of GnRH and/or at the pituitary to increase sensitivity of gonadotropes to GnRH (Imakawa et al., 1986; Baratta et al., 1994).

This array of factors demonstrates the importance of LH priming of an ovulatory follicle. Similar results were observed in anestrous ewes that have been progesterone primed. In this model the plasma levels of LH preceding the preovulatory surge of LH are an important determinant of follicular maturation as judged by subsequent corpus luteum function. However, administration of LH with different secretory patterns (infusion at a constant rate or pulsatile administration), did not affect the steroidogenicity of the subsequent CL (McNatty et al., 1981).

The duration and intensity of the pre-ovulatory surge of LH also is essential for the differentiation of a healthy follicle into a functional corpus luteum (Chapter 4; Chapter 6). When a first wave dominant follicle is induced to ovulate with a GnRH-agonist or hCG on d 5 of the estrous cycle an accessory corpus luteum is formed (Chapter 4). However, hCG with a longer half-life induced formation of a heavier and more functional accessory corpus luteum (Chapter 5). This effect is due likely to both persistence of hCG in the blood stream for an extended period of time (66 h, Chapter 4) but also extended binding of hCG to LH receptors. Mock and Niswender (1983) reported that times for internalization and degradation of radiolabeled hCG by ovine luteal cells were extended greatly when compared to ovine-LH. This extended binding of hCG to the membrane of luteal cells could be responsible for the increased stimulation of the follicle and growing CL.

Finally a more robust surge of LH (Chapter 6) is probably essential in triggering the cascade of events that leads to formation of a steroidogenic CL. The effect of differences in duration of the pre-ovulatory surge of LH have not been studied in cattle. In primates, the normal pre-ovulatory surge of LH lasts for a minimum of 48 h to 50 h (Hoff et al., 1983). When ovulation was induced with GnRH or a GnRH-agonist that induced a 11 h surge of LH, corpora lutea with suboptimal progesterone secreting capacities were formed. However, hCG with a half-life of 3 to 4 days, induced formation of a steroidogenic CL in primates (Zelinski-Wooten et al.,1991). After injection of 3000 IU of hCG in cattle, plasma concentrations of hCG were elevated markedly for 30 h and had not returned to baseline concentrations at 66 h after treatment. Therefore slow clearance of hCG in cattle is in accordance to what has been observed in humans (Yen et al., 1968).

In comparison, administration of 10 µg of Buserelin during the luteal phase of the estrous cycle in heifers induced an elevation of LH in serum for approximately 5 to 6.5 h (Chenault et al., 1990; Chapter 6). This differential exposures to LH or LH-like activity between injections of GnRH-agonist or hCG may result in differentiated development of the accessory CL.

Finally an essential physiological window of activity for LH may be represented by the period between the preovulatory surge and the ovulation of the follicle (Chapter 4 and Chapter 6). The importance of this window was more evident by the absence of LH priming of the follicle prior to injection of GnRH-agonist. During this period, additional LH stimulation of the follicle may be necessary for completion of ovulation and formation of a CL (Chapter 6).

In chapter 4 , administration of hCG at the time of a GnRH-agonist induced ovulation (Experiment 3) did cause an increase in subsequent luteal phase concentrations of plasma progesterone in some of the GnRH-agonist treated heifers. However, some heifers failed to have an enhanced secretion of progesterone. This is an additional indication of the potential role of LH stimulation during this transitory period following the pre-ovulatory surge of LH and prior to ovulation.

In summary, the peri-estrous follicular period of the estrous cycle can be divided into three periods: LH priming of the follicle, the pre-ovulatory surge of LH and the post-surge / pre-ovulation interval. Present studies provided evidence that the pre-ovulatory follicle has specific LH requirements during each one of these periods, regarding formation and function of the subsequent corpus luteum.

These observations are of importance when ovulation is induced for insemination at an appointed time in cattle. Since estrus is the combination of CL demise and the coordinated growth of an estrogenic follicle (Chenault et al., 1975, 1976; Thatcher and Chenault, 1976), a system that coordinates both follicular growth and CL regression should allow a more precisely synchronized estrus with good fertility.

Inducing a wave of homogenous follicular growth is essential to the timing of estrous occurrence (Chapter 3). The timed insemination protocol designed in Chapter 7 combined the effect of synchronizing follicular growth, coordinated regression of the CL and synchronization of ovulation. The use of a GnRH-agonist injection followed 7 d later by an injection of $PGF_{2\alpha}$ (Thatcher et al., 1989; Coleman et al., 1991; Twagiramungu et al., 1992; Wolfenson et al., 1994) has been used effectively to synchronize estrus. Synchronization of follicular growth contributed to a greater precision in timing of estrous behavior (Thatcher et al., 1989; Wolfenson et al., 1994) when compared to synchronization protocols based on the use of $PGF_{2\alpha}$ alone to control regression of the CL. More than 90% of the detected estruses occurred during the 4 d following injection of $PGF_{2\alpha}$ when the GnRH-agonist was administered 7 d before $PGF_{2\alpha}$ (Chapter 7, all control groups). The timed insemination protocol allowed 100% of the heifers to be presented for artificial insemination in 3 d versus 40 d for heifers of the control group (Chapter 7; Experiments 2 and 3) without a reduction in pregnancy rates.

An adequate strategy needs to be developed for providing the LH support to the ovulatory follicle, before, during and after the pre-ovulatory surge when developing a timed insemination program. Three pharmaceutical agents are available to induce ovulation in cattle.

In chapter 7, a GnRH-agonist was first chosen to induce ovulation in heifers and allow breeding on appointment. However, this treatment resulted in the occurrence of short cycles when the injection was given at 24 after $PGF_{2\alpha}$. Delaying the GnRH-agonist to 48 h after $PGF_{2\alpha}$ reduced the frequency of short luteal phases. The most plausible explanation is that the period of increased LH secretion during the 48 h after induction of CL regression (Chenault et al., 1976) induced a better differentiation of the growing follicle and therefore allowed the formation of a more functional CL among the population of heifers. Delaying the ovulatory injection to more than 48 h is not a perfect solution since most of the animals synchronized with this protocol are in estrus during the 3 days that follow the administration of $PGF_{2\alpha}$. Therefore some animals are in estrus before the ovulatory injection of GnRH-agonist.

An alternative would be to prevent ovulation between the time of CL regression and the ovulatory injection by inducing low levels of progestin (e.g., on additional hormonal manipulation) that would delay the occurrence of estrus but allow an increased LH priming of the ovulatory follicle (Savio et al., 1993a).

However, it was chosen to circumvent the absence of LH priming of the preovulatory follicle by inducing ovulation with an injection of hCG. When hCG was administered 48 h after ovulation, the occurrence of short estrous cycle was eliminated. The absence of LH priming that caused the occurrence of short cycles was compensated by the extended half life of hCG. This observation strengthened the role of LH support between the time of the preovulatory surge of LH and the time of ovulation (Experiment 6).

Implementing such a protocol could be useful in situations where estrus detection is not possible or difficult. However, the occurrence of estrus between the time of $PGF_{2\alpha}$ administration and the ovulatory injection of GnRH-agonist or hCG at 48 h is an imperfection of this treatment sequence since estrus detection cannot be eliminated totally.

When this protocol was implemented in lactating dairy cows during the period of January to May, 1995, the occurrence of short estrous cycles reported in heifers (Chapter 7) was not observed and this treatment improved pregnancy rates by more than 10% when compared to control cows (synchronization with GnRH-agonist followed 7 d later by $PGF_{2\alpha}$) during the warm months of April and May (W.W. Thatcher and J. Burke, personal communication). Thus, induction of ovulation with administration of a GnRH-agonist seems an adequate alternative in adult lactating dairy cows.

Lactating dairy cows develop larger follicles that are less estrogenic earlier during the preovulatory period, when compared to non-lactating cows (De la Sota et al., 1993). This may cause the preovulatory follicles to respond differently to a surge of LH induced by administration of a GnRH-agonist. The delayed growth of preovulatory follicles in non-lactating cows could explain the higher frequency of short estrous cycles observed in heifers treated with the timed-insemination protocol. For example, the second injection of the GnRH-agonist would induce a surge of LH too early in the development of some (± 15%) preovulatory follicles. This preovulatory follicle, due to inadequate maturation, would be unable to ovulate or would produce a corpus luteum with a reduced lifespan.

Perhaps hCG injected 36 h after administration of $PGF_{2\alpha}$ may completely eliminate the need for estrus detection without reduction of pregnancy rates in heifers. A limitation of this treatment is the immunogenicity of hCG . Repeated use of hCG in cattle can induce formation of antibodies that will neutralize the hCG molecule and dramatically reduce its binding to receptors (Sundby and Torjesen, 1978). Thus, it would be appropriate to develop a pharmacological delivery system, involving a more prolonged release of GnRH or a GnRH-agonist to potentially lengthen the period of LH secretion from the pituitary.

A normal period of LH secretion during the preovulatory surge of LH is 10 to 12 h (Chenault et al., 1975; Rahe et al., 1980) which is considerably longer than the 5 h surge elicited by an injection of GnRH or GnRH-agonist (Chenault et al., 1990). Insertion of a GnRH-agonist implant at the onset of estrus was associated with the development of a more functional CL, based on CL diameter, and increased luteal phase concentrations of progesterone in plasma during the subsequent cycle (Thatcher et al., 1993). Such implants are developed for inducing ovulation in the mare in which a prolonged surge of LH (2 days) is necessary for ovulation to occur

(Mumford et al., 1995). Current studies are testing the effect of a short term deslorelin (GnRH-agonist) implant on the follicular response, ovulation, and formation of an accessory CL in cattle (Thatcher and coworkers, on going studies).

Other pharmaceutical approaches can be utilized to induce ovulation. Estradiol, with its wide array of effects, given 24 to 48 hours after the luteolytic injection of $PGF_{2\alpha}$ may achieve a better synchronization of ovulation and differentiation of the ovulatory follicle into a functional CL. Ovulation can be induced with small doses (e.g., 400 µg) of estradiol after a synchronization protocol with two injections of $PGF_{2\alpha}$ given 11 days apart (Peters et al., 1977). In such a protocol, the injected estradiol may act synergistically with estradiol of follicular origin to better stimulate the hypothalamo-pituitary axis (Kesner et al., 1981; Baratta et al., 1994) and induce a more consistent and robust pre-ovulatory pattern of LH release.

Furthermore, estradiol may enhance the uterine release of $PGF_{2\alpha}$ and increase the percentage of animals undergoing complete luteolysis. This would achieve a consistent progesterone withdrawal that may result in a more homogeneous and rapid follicular growth.

Finally, estradiol may affect the ovulatory follicle by increasing the expression of LH receptors on granulosa cells (Richards, 1980) and stimulate mitosis of granulosa cells. However, more studies are necessary to understand the effects of estradiol used as an agent to control ovulation.

Conclusions: Several new directions should be considered to perfect existing protocols for estrus synchronization with the objective of timed insemination (Figure 8-1).

Chapter 3 showed the necessity to induce recruitment of a new follicular wave to improve conception rates by eliminating persistent follicles and inducing recruitment of an homogenous pool of dominant follicles across the treated population. Synchronization of follicular recruitment is essential to an estrus synchronization protocol.

Regression of the endogenous luteal structure should be controlled to allow rapid terminal growth and ovulation of the recruited follicle. Prostaglandin $F_{2\alpha}$ is used for this effect. Administration of a progestin implant at the time of follicle recruitment and administration of $PGF_{2\alpha}$ 48 h before removal of the implant enhances precision of estrus (Smith et al., 1984). It is possible that the increased LH pulsatility during the period between luteolysis induced by $PGF_{2\alpha}$ and removal of the progesterone / progestin implant, would permit a more homogenous growth of the pre-ovulatory follicle and a more homogeneous priming of the hypothalamo-pituitary axis with estradiol of follicular origin. Furthermore, $PGF_{2\alpha}$-induced regression of the CL allows a more consistent withdrawal from progesterone with removal of the implant. The combination of a progestin implant and the injection of $PGF_{2\alpha}$ may be necessary to closely control the final growth of the ovulatory follicle.

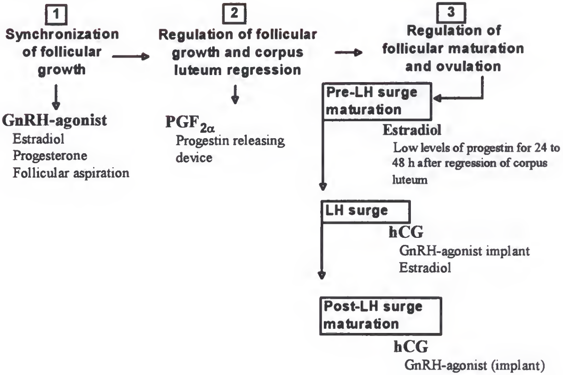

1
Synchronization of follicular growth
GnRH-agonist
Estradiol
Progesterone
Follicular aspiration
2
Regulation of follicular growth and corpus luteum regression
PGF2α
Progestin releasing device
3
Regulation of follicular maturation and ovulation
Pre-LH surge maturation
Estradiol
Low levels of progestin for 24 to 48 h after regression of corpus luteum
LH surge
hCG
GnRH-agonist implant
Estradiol
Post-LH surge maturation
hCG
GnRH-agonist (implant)

Figure 8-1. The three steps of an estrus synchronization program

Induction of ovulation should be implemented in a manner that allows an adequate LH priming of the ovulatory follicle. A GnRH-agonist or hCG, induced ovulation without full maturation of the follicle. The use of a short term implant of Deslorelin given in place of the GnRH-agonist or hCG ovulatory injection could maintain a longer stimulation of the pre-ovulatory follicle and growing corpus luteum

(W.W. Thatcher et al., ongoing research). Finally, estradiol can be used at low doses to enhance the physiological succession of events triggered by the pre-ovulatory follicle that are essential for follicular maturation.

Further research should focus on enhancing the maturation of the follicle in the periovulatory period and preventing expression of estrus and ovulation before the second injection of GnRH-agonist. Combining a small dose of progesterone in a long-acting (2 days) presentation with the luteolytic injection of $PGF_{2\alpha}$ could achieve this effect without increasing the number of interventions. This additional progestin would maintain low concentrations of progesterone for 48 h after administration of $PGF_{2\alpha}$ and prevent the occurrence of the pre-ovulatory surge while allowing LH support of the growing follicle.

Accumulation of treatments for efficient timed-insemination seems awkward. Furthermore, drugs used for experimentation (CIDR, PRID) are not currently approved by the Food and Drug Administration for use in cattle. The timed-insemination program described in this dissertation could be applied immediately, since GnRH-agonist, hCG and $PGF_{2\alpha}$ are available commercially in the United-States. Study of the economic feasibility of this timed-insemination protocol as well as its application to the lactating dairy cow is underway (W.W. Thatcher, R.L. de la Sota, J. Burke and M. Delorenzo).

Close control of follicular dynamics makes possible the development of a single device that would deliver in a sequential timely manner the necessary drugs for follicle recruitment, corpus luteum regression, follicular maturation and induction of ovulation at a fixed time. Development of such a system will be a future challenge for investigation and commercial companies.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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